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VOLUME LXVI
JANUARY TO JUNE, 1934

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN is issued six times a year. Single numbers, $1.75. Subscription per volume (3 numbers), $4.50.

Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa. Agent for Great Britain: Wheldon & Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W.C. 2.

Communications relative to manuscripts should be sent to the Managing Editor, Marine Biological Laboratory, Woods Hole, Mass., between May 1 and October 1 and to the Institute of Biology, Divinity Avenue, Cambridge, Mass., during the remainder of the year.

5106

Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

LANCASTER PRESS, INC., LANCASTER, PA.

CONTENTS

No. 1. February, 1934

PAGE

Loefer, John B.
The Trophic Nature of Chlorogonium and Chilomonas 1

Sayles, Leonard P.
Effect of Ringer's Solution on Tail-Regeneration in the Tadpole of Rana palustris 7

Coonfield, B. R.
Coördination and Movement of the Swimming Plates of Mnemiopsis leidyi, Agassiz 10

Rugh, Roberts
Induced Ovulation and Artificial Fertilization in the Frog 22

Parker, G. H., and Helen Porter
The Control of the Dermal Melanophores in Elasmobranch Fishes 30

Helff, O. M.
Studies on Amphibian Metamorphosis. XIII. Stability and growth of anuran tympanic membrane following larval involution 38

Harvey, E. Newton
The Air Turbine for High Speed Centrifuging of Biological Material, together with some Observations on Centrifuged Eggs 48

Chamberlain, Thomas K.
The Glochidial Conglutinates of the Arkansas Fanshell Cyprogenia aberti (Conrad) 55

Dawson, Alden B.
The Colored Corpuscles of the Blood of the Purple Sea Spider, Anoplodactylus lentus Wilson 62

Kidder, George W.
Studies on the Ciliates from Fresh-water Mussels, I. 69

43249

No. 2. April, 1934

Harvey, E. Newton
Biological Effects of Heavy Water 91
Mitchell, Philip H., and Julian P. Solinger
The Effects of Land Drainage upon the Excess Bases of Sea Water 97
Chao, Iping
Paradox Phenomena in the Cardiac Ganglion of Limulus polyphemus 102
Reznikoff, Paul, and Dorothy Grout Reznikoff
Hematological Studies in Dogfish (Mustelus canis) 115
Barnes, T. Cunliffe
Further Observations on the Salt Requirements of Ligia in Bermuda 124
Chesley, Leon C.
The Concentrations of Proteases, Amylase, and Lipase in Certain Marine Fishes 133
Whiting, P. W.
Egg-Trinuclearity in Habrobracon 145
Whiting, P. W., Raymond J. Greb, and B. R. Speicher
A New Type of Sex-Intergrade 152
Bostian, C. H.
Biparental Males and Biparental Ratios in Habrobracon 166
Trager, William
The Cultivation of a Cellulose-Digesting Flagellate, Trichomonas termopsidis, and of certain other Termite Protozoa ... 182
Svedberg, The, and Astrid Hedenius
The Sedimentation Constants of the Respiratory Proteins 191
Schmuck, M. Louise
The Male Somatic Chromosome Group in Sciara pauciseta ... 224
Harvey, Ethel Browne
Effects of Centrifugal Force on the Ectoplasmic Layer and Nuclei of Fertilized Sea Urchin Eggs 228

No. 3. June, 1934

Dan, Katsuma
Electrokinetic Studies of Marine Ova. II. Cumingia tellinoides, Asterias forbesii, Echinarachnius parma, Nereis limbata, and Cerebratulus lacteus 247
Costello, Donald P.
The Hyaline Zone of the Centrifuged Egg of Cumingia 257
Heilbrunn, L. V.
The Effect of Anesthetics on the Surface Precipitation Reaction 264
Howland, Ruth B., and Alan Bernstein
H-Ion Concentration as a Factor in the Toxicity of Amines for Amoeba proteus 276
Kidder, George W.
Studies on the Ciliates from Fresh Water Mussels. II. The nuclei of Conchophthirius anodontae Stein, C. curtus Engl., and C. magna Kidder, during binary fission 286
Coe, Wesley R.
Analysis of the Regenerative Processes in Nemerteans 304
Birnie, James H.
Regeneration of the Tail-Fins of Fundulus Embryos 316
Roudabush, Robert L.
Present Status of Longitudinal Division in Hydra 326
Chesley, Leon C.
The Influence of Temperature upon the Amylases of Cold- and Warm-blooded Animals 330
Welsh, John H.
The Structure and Reactions of the Tentacles of Terebella magnifica W. 339
Welsh, John H.
The Concentration of Eosin and the Photodynamic Effect on Tentacles of a Terebellid Worm 346
Glaser, Otto, and Edmund Piehler
The Mobilization of Calcium during Development 351
Hamlett, G. W. D.
Nema Parasites in Embryo Bats 357
Lillie, Ralph S.
The Influence of Hypertonic and Hypotonic Sea Water on the Artificial Activation of Starfish Eggs 361

Vol. LXVI, No. 1 February, 1934

THE
BIOLOGICAL BULLETIN

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THE TROPHIC NATURE OF CHLOROGONIUM AND CHILOMONAS[1]

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Introduction

The application of pure culture methods has done much to further our knowledge of protozoan nutrition. Growth of organisms in synthetic media has been accomplished largely through a process of trial and error, and very often success has been attributable to the wide range of adaptability which many of these forms exhibit. Some of the green flagellates may be either completely autotrophic in light, utilizing inorganic nitrogen and carbon dioxide, or totally heterotrophic in darkness. While some of the plant-like flagellates in particular are capable of existence under such extremely varied conditions, the great majority of protozoa are restricted to a more limited food environment. The purpose of the following experiments is to determine the trophic nature of *Chlorogonium euchlorum* Ehrenberg, *C. elongatum* Dangeard and *Chilomonas paramecium* Ehrenberg when grown in bacteria-free cultures, *i.e.*, to determine their capability of autotrophic existence in light and their heterotrophic nutrition in darkness. *Chilomonas,* being colorless, normally assimilates soluble organic food, although claims for its completely autotrophic nature have recently been advanced by Mast and Pace (1932). Jacobsen (1910), working with *Chlorogonium euchlorum* in bacterized cultures, believed this form to be autotrophic in light as well as heterotrophic in darkness, although he was unable to control cultural conditions adequately to determine this fact with certainty. Grateful acknowledgment is due Professor R. P. Hall for suggestions during the investigation.

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[1] Part of a thesis submitted in partial fulfillment of the requirements for the degree of doctor of philosophy at New York University, June, 1933.

Material and Methods

Pure lines of *C. euchlorum* and *C. elongatum* were obtained from the German University at Prague through the courtesy of Professor E. G. Pringsheim, while the bacteria-free strain of *Chilomonas paramecium* was derived from a single organism isolated from pond water at Woods Hole during the summer of 1932. Cultures of the three species were maintained in culture tubes in a 0.25 per cent tryptone-salt medium at pH 7.0.

Before it can be concluded that a certain nutritional factor in a given medium is or is not utilized by the organism concerned, it is necessary, according to Lwoff (1932), to subculture that species at least three or more times in order to eliminate growth effects produced by compounds present in the original stock medium transferred with the inoculum and in order to allow for utilization of reserve food materials. Accordingly, conclusions stated below are based only on cultures which have been carried through a number of transfers, the cultures having been maintained in constant light at a temperature of 28° C. in a thermostat-controlled water bath. In each series of transfers, 6–12 culture tubes containing the same volume of medium were inoculated equally from a dilution flask (Loefer, 1934). After incubation transfers to similar media were made; this procedure was continued until growth ceased or until the possibility of continued growth was established. The following media at pH 7.0 were used in the respective experiments designated later.

Medium A:

NH_4NO_3	0.5 gram
KH_2PO_4	0.5 “
$MgSO_4$	0.1 “
NaCl	0.1 “
$FeCl_3$	trace
Distilled water	1 liter

Medium B:

KNO_3	0.5 gram
KH_2PO_4	0.5 “
$MgSO_4$	0.1 “
NaCl	0.1 “
$FeCl_3$	trace
Sodium acetate	2.5 gram
Dextrose	2.0 “
Proteose-peptone	2.5 “
Distilled water	1 liter

Medium C:

KH_2PO_4	0.5 “
$MgSO_4$	0.1 “
NaCl	0.1 “
$FeCl_3$	trace
Sodium acetate	2.5 gram
Distilled water	1 liter

Bacteriological tests for purity of the cultures were carried out at regular intervals during the course of each experiment. The sodium acetate used was obtained from the Eastman Kodak Co., Rochester, N. Y., while tryptone, peptone, and dextrose were products of the Difco Laboratories.

Autotrophic Nutrition

Tubes containing medium A were inoculated with *C. euchlorum* from a dilution flask and placed at a north window at room temperature. At the end of four weeks the cultures were intensely green. Consecutive transfers were made at four-week intervals for forty weeks, at the end of which time it was evident that autotrophic existence in light is possible indefinitely in the medium used. Homologous series on *C. elongatum* showed the same result.

The object of another group of experiments was to determine whether growth is more rapid in a solution containing nitrates or in one containing ammonium compounds. For this purpose medium C was used as a base. Equal lots containing 0.5 per cent concentrations of NH_4NO_3, KNO_3, NH_4Cl and $(NH_4)_2SO_4$, respectively, were tubed and sterilized in the regular manner. After inoculation (1.0 cc.) with *C. euchlorum,* cultures were grown at room temperature near a north window. After fifteen days no significant differences in growth were evident, as determined by the Sedgwick-Rafter counting-cell method. Four successive transfers were made in each type of medium; subsequent growth indicated that this species uses either nitrate or ammonium to equal advantage. The results for *C. elongatum* were similar. No appreciable pH changes occurred during the culture periods.

Chilomonas paramecium has been reported capable of using ammonium nitrogen (Mast and Pace, 1932). In an attempt to verify this observation the same types of media as were used above for *Chlorogonium* were inoculated with *Chilomonas.* A number of the cultures were kept at room temperature and some at a constant temperature of 28° C. in moderate light. Growth occurred in all of them after four or five days. A second transfer was made, but only slight growth was noted in a few of the tubes after an equal period of time. No growth took place at all in cultures of the third transplant, even after a longer period of time. Even when these media contained 0.05–0.10 per cent Na_2SiO_3 in addition to the regular constituents, *Chilomonas* did not survive a third transfer. *Chlorogonium,* likewise, will not grow in darkness on inorganic nitrogen, even in the presence of sodium acetate and silicate.

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Heterotrophic Nutrition

Since it was impossible to grow *Chlorogonium* in darkness in a medium containing inorganic nitrogen only, a medium containing several different carbon and nitrogen compounds was used (medium B). Sterile tubes of this solution were inoculated with *C. euchlorum* on November 10, 1932, and incubated in total darkness at room temperature. Subinoculations were made at intervals of approximately four weeks, and to date the organisms have been transferred 11 times and the cultures are still green, indicating that growth and continued synthesis of chlorophyll is taking place. Parallel experiments on *C. elongatum* indicate the same result. Although the organisms appear somewhat smaller than when grown in the usual stock medium in light, their normal color and continued division indicate that indefinite growth without photosynthesis is possible when the culture medium contains adequate nitrogen and carbon sources.

Discussion

In the group of chlorophyll-bearing flagellates, most species utilize either nitrate or ammonium nitrogen in light, using CO_2 as a carbon source. Among the Phytomonadida which are facultatively autotrophic, various nitrogen sources produce a differential effect in amount of growth. Lwoff (1932) found that *Hæmatococcus pluvialis* grew better with ammonium salts than with nitrates, although *Chlamydomonas agloëformis* grew equally well in either medium. The group Euglenida likewise exhibits specific growth differences (Dusi, 1933), some forms (*Euglena gracilis, E. stellata, E. klebsii*) growing equally well with ammonium salts or nitrates as nitrogen sources. *E. anabæna* is more limited since it is unable to utilize nitrate, but grows well on ammonium salts. Others (*E. pisciformis, E. deses*), although generally considered totally photosynthetic organisms because of their green color, are actually mixotrophic since they cannot utilize inorganic nitrogen. These few instances are indicative of the variations found among chlorophyll-bearing flagellates. The results obtained for *Chlorogonium* (*euchlorum, elongatum*) mark them as truly autotrophic species since they can utilize both ammonium and nitrate nitrogen. The wide range of adaptability of this genus, shown by its ability to grow heterotrophically as well as autotrophically, no doubt accounts for Jacobsen's (1910) conclusion that it was primarily mixotrophic rather than autotrophic in its food habit.

Only in one case has truly autotrophic nutrition been claimed for a colorless flagellate. Mast and Pace (1932) state that, " Chilomonas can consequently, without light, synthesize carbohydrates, fats, proteins, and protoplasm from inorganic compounds, obtaining nitrogen from NH_4 and carbon from CO_2." Such synthesis is effected in an inorganic salt medium containing Na_2SiO_3 either with or without sodium acetate. In the present investigation with bacteria-free cultures of *Chilomonas paramecium* there was no indication that inorganic nitrogen could be utilized, with or without Na_2SiO_3 in the medium, even when sodium acetate was supplied as an organic carbon source. Growth which occurred in the first and second subcultures was probably due to the small amount of organic nitrogen transferred with the inoculum from the stock medium. Inasmuch as these results have been reported only in abstract form, it is at present impossible to repeat their experiments on this strain, using their exact method.

It would appear (Cleveland and Collier, 1930) that certain strains of *Leptomonas* can be cultured in dextrose and other carbohydrate solutions which contain no nitrogen source. From the description of the methods used, it seems highly probable that the small amount of nitrogen necessary for growth was transferred from the stock medium with the inoculum. It is extremely doubtful that these forms would have survived successive transfers in this medium, since growth without nitrogen is unknown in any other protozoa.

Under favorable conditions of organic nutrition many green, otherwise autotrophic, organisms have been cultivated in total darkness for varying periods of time. Jacobsen's (1910) results on *Chlorogonium* were mentioned above. A race of *Euglena gracilis* has been grown saprophytically for several years in total darkness by Lwoff (1932), having, however, lost its green color in the meantime. *Chlorogonium* (*euchlorum, elongatum*) differs in that it still retains its green color after a year of cultivation in total darkness, although the chloroplasts appear somewhat modified.

SUMMARY

Chlorogonium (*euchlorum, elongatum*) is capable of indefinite autotrophic growth in a mineral medium in light, growth in nitrate being as good as in cultures containing ammonium salts. It was impossible to maintain bacteria-free cultures of *Chilomonas paramecium* indefinitely by subculturing them at regular intervals in a medium containing inorganic nitrogen, even in the presence of sodium acetate as a carbon source. Since November 10, 1932, both species of *Chlorogonium* have been cultured in total darkness in a medium containing mineral salts, sodium acetate, dextrose and proteose-peptone without losing their green color.

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EFFECT OF RINGER'S SOLUTION ON TAIL-REGENERATION IN THE TADPOLE OF RANA PALUSTRIS

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It has been shown that the regenerative activity of the fresh-water oligochæte *Lumbriculus* is decreased in balanced salt solutions (Ringer) which are isotonic, or nearly so, with the body fluids of that worm (Sayles, 1928). Also, in the case of certain marine invertebrates, it has been found that dilutions of the sea water increase the rate of regeneration (Loeb, 1892; Goldfarb, 1914; Lloyd, 1914; Keil, 1932 and others). In the case of marine forms, of course, the body fluids are approximately isotonic with undiluted sea-water.

With these facts in mind a study was made to determine whether or not a similar slowing-up of regeneration in isotonic salt solutions might occur in the case of frog tadpoles.

Tadpoles of *Rana palustris,* varying in total length from 40 to 65 millimeters and with small posterior legs, were used. They were first anæsthetized in a solution of approximately 1 part of chloretone in 2,200 of water. Either 15 or 20 mm. of the tail were then removed.

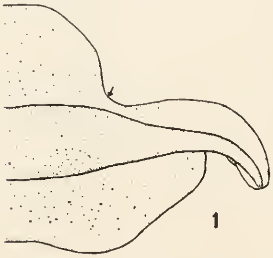
The first experiments on this material were carried out during the summer of 1927. Then, in 1929 and again in 1932, results essentially the same as those of the first year were obtained.

The results of two experiments, typical of a number performed, may be presented to indicate the effect of Ringer solutions on the length and form of the bud of new tissue. In one instance, four tadpoles were cut. Tadpole *A* (52 mm. total length) was placed in water. *B* (55 mm. long), *C* (50 mm. long), and *D* (40 mm. long) were placed in buffered frog Ringer. All four had 15 mm. of tail removed. After two weeks the lengths of the new buds were as follows: *A,* 9.0 mm.; *B,* 1.9 mm.; *C,* 3.6 mm.; *D,* 3.4 mm. All of these were killed at the end of the third week, at which time no marked change in the relative lengths of the buds had occurred.

In another experiment four tadpoles, each 40 mm. in total length, had 20 mm. removed from the tail. *A* was placed in tap-water; *B* in Ringer of 0.5 usual strength; *C* in ordinary frog Ringer; and *D* in Ringer of 1.1 usual strength. After twenty-three days the lengths of the buds were as follows: *A,* 12.3 mm.; *B,* 9.3 mm.; *C,* 7.1 mm.; *D,* 6.4 mm.

In all individuals regenerating in tap-water the tail bud is complete with well-formed swimming membrane. Tadpoles regenerating in 0.5 Ringer usually produce complete tails of the ordinary type. In some cases, however, the musculo-skeletal axis is complete but the caudal membrane is much reduced or even absent in part of the bud. A regenerate of this type is shown in Fig. 1.

In straight frog Ringer about two-thirds of the short buds have well-developed membranes. These apparently differ from the control animals only in the length of the bud. In two of these tadpoles, however, the buds formed have only narrow, somewhat irregular portions of the membrane (Fig. 2). In the cases of the other tadpoles regenerating in this solution, however, a slender bud is formed with little or no caudal membrane. Figure 3 shows such a regenerate. This particular bud is of interest, too, because of the fact that there are, at the posterior tip, two very small, thin pieces of tissue which resemble the caudal membrane.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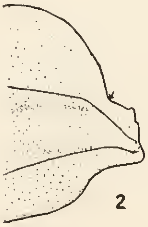
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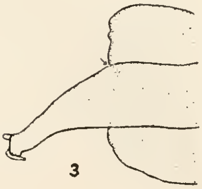
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FIG. 1. A tail bud formed after 11 days by a tadpole regenerating in 0.5 Ringer solution. × 4.

FIG. 2. A tail bud formed after 29 days of regeneration in straight Ringer solution. × 3.

FIG. 3. Slender bud consisting merely of musculo-skeletal axis with two very small pieces of caudal membrane at posterior tip. A regenerate of 11 days in straight Ringer solution.

In each of the above figures an arrow indicates the transition zone between old and new tissues.

In a few instances tadpoles have lived and regenerated in 1.1 Ringer solution. In each of these cases the bud formed is without the caudal membrane.

In this connection it is interesting to note the report of Avel (1932) that, with the approach of metamorphosis, ability to regenerate becomes less and then disappears in the caudal membrane while at corresponding levels it persists for a long time in the musculo-skeletal axis. No experiments have been carried out to determine whether or not the isotonic solution affects, in a similar manner, all stages in the development of tadpoles. In the present work, however, all control individuals produced well-formed tails with complete caudal membranes. Thus it is quite clear that the results here described are due to the effect of the Ringer solution and not to the approach of metamorphosis alone.

SUMMARY

Tadpoles of *Rana palustris*, regenerating new tails, produce, in general, less new tissue when kept in an approximately isotonic frog Ringer solution than when allowed to remain in ordinary fresh water.

The regeneration of the caudal membrane is frequently affected by isotonic solutions. Occasionally the bud of new tissue consists merely of the main axis without any membrane. In some other cases a much reduced or irregular membrane is formed.

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COÖRDINATION AND MOVEMENT OF THE SWIMMING-PLATES OF MNEMIOPSIS LEIDYI, AGASSIZ

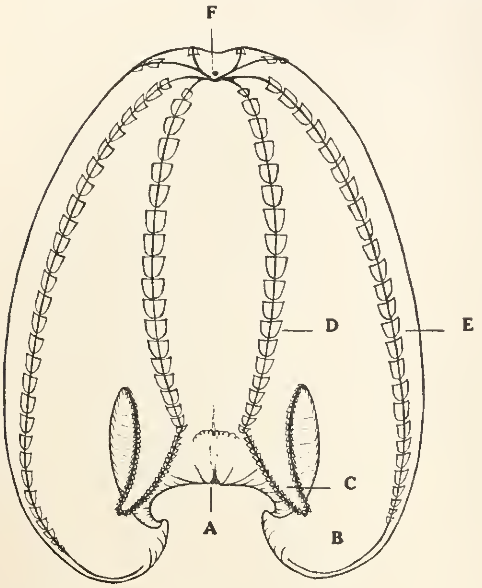
B. R. COONFIELD

(From the Biological Laboratory, Brooklyn College, and the Marine Biological Laboratory, Woods Hole)

The synchronized movements of the swimming plates of *Mnemiopsis leidyi* in a natural environment indicate the presence of a coördinating system. The rhythm of the plate movement in the rows shows the effect of a coördinating mechanism rather than demonstrating how each plate is stimulated. A single isolated plate containing only a very small amount of protoplasm at its base will beat rhythmically for several hours. This shows that each plate possesses a certain amount of independence, but this independence is overcome by a coördinating system when the body of *Mnemiopsis* is intact. Although Lillie (1906) reported that isolated plates of *Eucharis* continue to beat for several hours and Verworn (1890) recorded the same for *Cestus,* I am convinced that the full significance of this activity has not been realized. It was the persistent beating of these isolated plates, both complete and fragmented, that led me to believe that the transmitting and coördinating system of *Mnemiopsis* has not been fully understood. These experiments have been done, therefore, to add to our knowledge of the transmitting and coördinating mechanism in *Mnemiopsis.*

Ctenophores are biradially symmetrical animals having distinct oral and aboral ends. *Mnemiopsis* is a member of a group of ctenophores which have characteristically a pair of lobes protruding forward beyond the mouth (Fig. 1). This animal moves along in the water just below the surface with its oral end in advance, propelling itself by the successive movements of paddle plates which beat in an aboral direction. Each paddle plate consists of several very long cilia fused and beating as a unit. The plate nearest the aboral end beats first, followed by each plate in succession toward the oral end of the animal (Fig. 3). According to Parker (1905*a*), this type of movement is known as metachronism. The metachronal wave of *Mnemiopsis* is aboral to oral while its effective plate beating is oral to aboral. The plates are arranged on the body of the animal in two opposite pairs of long rows which are in the esophageal plane and are called adesophageal and in two opposite pairs of short rows which are in the tentacular plane and are called adtentacular. On the basis of coördination of the plate movement the rows fall naturally into quadrants each consisting of an adesophageal row and an adtentacular row. The coördination of the two plate rows of a quadrant is quite definite for *Beroe* (Chun, 1880) and for *Pleurobrachia* (Child, 1933). Although the coördination in each quadrant is definite in *Mnemiopsis,* usually all eight rows are synchronized.

That each row of plates in ctenophores represents a transmission path along which stimuli flow to initiate the plate movements is a common belief. This path has been regarded by a few investigators as representing a nervelike, transmitting element not yet differentiated into true nervous tissue but remaining neuroid in form. Bauer (1910) and Bethe (1895) believed the path to be nervous. By staining *Cydippe* with



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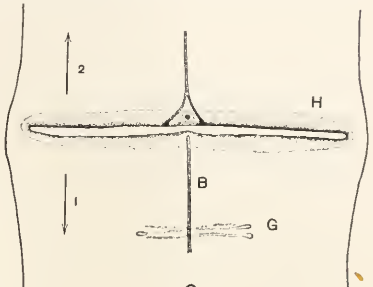

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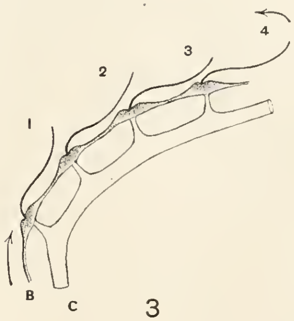

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FIG. 1. A diagram of *Mnemiopsis*. *A*, mouth; *B*, oral lobe; *C*, auricle; *D*, adtentacular swimming plate row; *E*, adesophageal swimming plate row; *F*, apical sense organ in the aboral zone.

FIG. 2. A camera-lucida drawing of a *Mnemiopsis* swimming plate stained with methylene blue; *B*, interplate structure; *G*, muscle cells; *H*, swimming plate base; *1*, arrow pointing in the aboral direction; *2*, arrow pointing orally and in the direction of the metachronal wave.

FIG. 3. A diagram of a swimming plate row showing only a few plates. These plates beat in succession as indicated by the numbers, *1*, *2*, *3*, and *4*. This sequence of movement is known as the metachronal wave. The arrow near *4* indicates the effective stroke direction. The interplate structure is shown at *B*, and the food canal at *C*.

methylene blue and later sectioning the tissue, Bethe found a network of nerves. According to Göthlin (1920), in *Beroe* the activating impulses arise in the nervous system and are conducted by the nerve net to the swimming plate. Ater the impulse reaches the plate it is then transmitted from cell to cell along the plate row. Parker (1905*a*) and Child (1933) believed the initial stimulus in *Pleurobrachia* to come from the sense organ in the aboral zone and spread to the plate rows. The paddle plates of *Mnemiopsis* show definitely a certain amount of coördination. When coming in contact with an object, the body wall contracts, and at the same time the paddle plates cease to beat. The plates resume beating soon afterward and usually by a spreading of their movement from the aboral to the oral zone. This indicates that the center of coördination is in the aboral zone.

In considering the interpretation of the experiments reported here, the following questions arise. Is the aboral sense organ the center of coördination? Does the coördination in each plate row depend entirely upon the stimuli arising in the aboral center? Will this sensory center exhibit its dominance during all rates of plate movements? Is this stimulating element in each plate row strong enough to resist any reversed impulses? Does each plate row exhibit an aboral dominance as reported by Child (1917)? Is the transmitting element nervous or is it less differentiated than nervous tissue? These questions are considered in this report.

Materials and Methods

Mnemiopsis were unusually numerous at Woods Hole from middle August until late September during the summer of 1933. This was due to a wind from the southeast which continued blowing for several days. Uninjured specimens of these ctenophores in stages from late embryos to adults were collected and kept in large finger bowls in running water in the laboratory. Specimens will live in the laboratory for several days only if they are kept in running water. Those of intermediate size were usually selected for observation to determine plate row coördination, while in the experiments in which the plate rows were removed, both large and small animals were used.

The experiments were of three types. In the first type, both large and small pieces of plate rows were stained with methylene blue. In the second type, the action of swimming plate rows was interrupted either by cutting out plates or by holding a plate. In the third type, observations were made on excised pieces of the plate rows.

The Staining Effect of Methylene Blue

The main structures around the sensory body and also those leading from it to the plate rows have been described for *Pleurobrachia, Beroe,* *Bolinopsis,* and *Mnemiopsis* by Child (1933). Only the transmitting elements in the plate rows are to be considered in this section of this report. Parts of the swimming plate rows of *Mnemiopsis* were stained in these experiments. This animal is quite sensitive to methylene blue and will fragment if subjected to other than very dilute solutions. This is in accord with the report of Child (1933). Sections of the swimming plate rows were cut from the animal with small scissors and stained from 2 to 12 hours in a solution of methylene blue of about 1 to 500,000 parts of sea water. These stained pieces of swimming plates were examined in a living condition under both high and low magnifications. The stain showed a structure which connected the plate bases in a row. This interplate structure is a narrow band of granules and is uniform in width except at its enlarged aboral end which contains a small distinct granular spot. The oral end of this band extends into the base of the next plate where it ends clearly without any granular connection to the plate base (Fig. 2). The interplate band lies above and is not connected to the food canal (Fig. 3). Child (1933) has reported the presence of this interplate structure in *Bolinopsis* and in *Mnemiopsis* but he made no mention of the small granular spot.

Although having stained numerous pieces of plate rows of *Mnemiopsis* in methylene blue, I have never found any cellular structure either in these interplate bands or in the plate bases. The only cells brought out by the stain were those of a few muscles lying parallel to and midway between the plates. All other structures shown by the stain were quite granular. The suggestion made by Child that these interplate bands are conducting paths must rest on the position of these bands rather than on their structure. The fact that they point in the direction of transmission may also have influenced Child in making his conclusion. It is clear to me that neither the position of the interplate structures nor their morphological polarity gives sufficient proof of this nerve-like interpretation.

Experiments on the Plate Rows on the Body of Mnemiopsis

The movements of the eight paddle plate rows of ctenophores usually are synchronized. Coördination between two quadrants is more easily destroyed than between the two rows of a single quadrant. Rhythm in a single row is more persistent than it is in a single quadrant. Even this rhythm of the paddle plates in a single row can be temporarily destroyed in *Mnemiopsis* by cutting across any of its plate rows. Very soon, however, rhythm is reëstablished. In one experiment the rhythm in a row was reëstablished within 5 minutes after the cut was made. Usually, when a cut is made, all beating in a row ceases. Then the two rows in a quadrant beat independently for a short time. After this short interval the rhythm in the quadrant is reëstablished. Parker (1905*a*) touched a plate row of *Mnemiopsis* with a pointed instrument. The temporary cessation of beating of the two plates touched was due to the contraction of muscles in the outer body wall closing the plate groove at that point, thus causing a mechanical paddle plate block. When the stimulus was removed, the muscles relaxed and the blocked plates resumed beating. Only the blocked plates failed to beat, while rhythm in the row was not interrupted. Parker held this to be proof that the impulses along the plate row were not aided by the mechanical effect of the paddle plates striking one another as they contract. I verified this experiment by holding a single plate in a row with a stiff hair and found that the rhythm in the row was not interrupted, although the plate which was held did not beat.

The auricles have a row of paddle plates continuous from the adtentacular row to their bases at the oral lobes. These long plates are set very close together (Fig. 1). Each plate touches the one in front when completing its effective stroke and strikes also the one behind in its return stroke. The plates in the adesophageal and the adtentacular rows do not strike one another so effectively. Here was an opportunity to test whether transmission in the row is aided by one plate touching another in beating. Two or three plates were held firmly with a bristle. The action of the other plates was not interrupted in the least. The auricle was then cut across a paddle plate row to a point about midway through the jelly. The cut ends of the plate row were pulled apart by the contracting muscles. Thus a complete gap was formed across the plate row. Beating in the row ceased at first but soon resumed with its usual rate and rhythm. The cutting of either an adesophageal or an adtentacular plate row caused a cessation of plate beating in that row for a brief interval. Almost immediately the rhythm was reëstablished in the plate row followed by that in the quadrant.

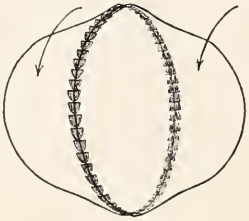
The fact that holding a single plate does not interrupt the rhythm in the plate row shows that transmission does not depend upon movement from plate to plate. This suggests the presence of a real physiological transmitting element. The cutting across any of the plate rows brought about a sudden cessation of plate movement, which suggests that the transmission path is in the plate row. Following the cutting, the rapid recovery of plate movement in the row with the reëstablishment of rhythm indicates that the transmission path is quickly reformed. The question then arises as to whether this transmission path is nervous or is more simple and less differentiated. The rapid recovery of rhythm in the row reveals the more simple nature of this system. The reaction of plates on excised pieces of rows described in the following section also confirms this conclusion.

Experiments on Excised Pieces of Swimming Plate Rows

The beating of the isolated and fragmented plates collected in tow suggests their independence of other paddle plates. This independence was observed in *Eucharis* and reported by Lillie (1906). He stated that quite often the rhythm in the isolated pieces approximated that of the plates in the normal animal. Lillie (1908) cut strips of jelly containing portions of swimming plates from *Eucharis* and from *Mnemiopsis.* According to his observations, soon after the removal, the beating in the excised pieces returned to normal. In my experiments on *Mnemiopsis* the plate activity ceased at once in the strips of plate rows as they were removed. Very soon, however, the beating in these strips was resumed and the waves passed from the aboral end to the oral end. Verworn (1890) reported that a single isolated plate of *Cestus* will continue to beat if it has a small amount of protoplasm attached. It is therefore perfectly clear that swimming plates will beat rhythmically even if they are detached from the ctenophore body. Will isolated plates respond differently to those located normally on the animal?

Sections of both the adesophageal and adtentacular swimming plate rows were cut out of *Mnemiopsis.* Some of the pieces were half of a row in length and others were longer or shorter. Although movement ceased during the cutting, activity was resumed in the pieces within 10 minutes after their excision. Both the effective stroke and the metachronal wave were the same as before the pieces were removed. The rhythm was unchanged and, although the rate was reduced at first, the normal speed was soon regained. Within one hour each piece had formed a complete compact body. In general, three types of bodies were formed by these excised pieces. When a small amount of jelly was removed with approximately one-half of a plate row, the reformed body was egg-shaped. The plate row was oriented midway around the mass on its shortest axis (Fig. 4). Practically the same results were obtained when a larger amount of jelly was removed with half a plate row, though the jelly in this case did not completely fuse at its cut ends (Fig. 5). In both these cases, the ends of the paddle plate rows always fused perfectly. When a still larger amount of jelly was removed with one-half a plate row, the reformed body was more elongated and the cut ends of the jelly were less completely fused than in the previously described cases. This incompletely fused jelly left a cavity large enough to prevent the fusing of the ends of the plate row (Fig. 6). All pieces of jelly containing paddle plates lived and continued their beating for two weeks. They were then thrown away, though still in good condition.

The effective beating of the paddle plates in all of these cases caused the individual pieces to rotate. Mechanically, the rotation must be directly opposite to the direction of effective beating of the swimming plates. Hence, by observing the direction of movement of the rotating pieces, the direction of the effective paddle plate beating can be determined. If a revolving excised piece of swimming plate is touched anywhere with a needle, the whirling stops at once, but the plate beating is resumed in a short time. Often this resumed paddle plate action causes the whole piece to rotate in the opposite direction, giving definitely a demonstration of reversed ciliary beating in *Mnemiopsis.* This reversal may continue until a dozen revolutions have been made. Then, without being stimulated further, all movement ceases for an instant and the regular beating follows. This reaction is common in all three types of reformed plate rows. I have never seen a metachronal wave reversal



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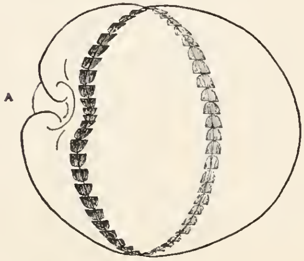

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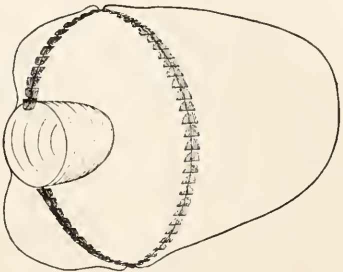

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Fig. 4. This shows the rounded jelly and the swimming plate row after its cut ends have completely fused to each other. The arrows indicate the direction in which the piece rotates.

Fig. 5. At *A* the incomplete fusion of the jelly is shown. The swimming plate row ends have fused to each other completely.

Fig. 6. This illustrates the incomplete fusion of the jelly and of the cut ends of the swimming plate row. The direction of rotation in both Figs. 5 and 6 is the same as in Fig. 4.

in any of the pieces. Moreover, a reversal of metachronism in a normal plate row of *Mnemiopsis* has never been reported. Parker (1905*a*) noticed an apparent reflection of the wave as it reached the oral end of a plate row in *Pleurobrachia*. This reflection wave rarely traveled aborally the entire length of the plate row.

Both large and small individuals were cut transversely midway between their aboral and oral ends. Immediately following the cutting the paddle plates in both halves stopped beating. The plate action was resumed in the aboral halves within 10 minutes and the rhythm was reestablished at once. Beating was not resumed in the oral halves until a few minutes later, and then action of the paddle plates in these halves was not synchronized as in the aboral pieces. The plates beat slowly at first and quite independently of one another. Within 24 hours the normal rhythm had been established in these oral halves, however. In one oral half, a complete aboral end had been reformed within 48 hours. This reformed zone was perfect, including the sense organ and food canal connections from the stomach to the paddle plate rows. More experiments of this nature have been planned.

The definite polarity in the intact plate rows of *Mnemiopsis* indicates a higher type of transmitting system, but when the polarity is destroyed in excised pieces of these plate rows, the more elementary nature of this system is revealed. Furthermore, the ability of the jelly and the transmission system to reform quickly following a cut indicates that both of these structures are not very highly differentiated. Therefore, these experiments on excised pieces of plate rows of *Mnemiopsis* show that its transmitting system is simple and elementary in nature.

Discussion

According to those who have studied stained sections and stained regions of ctenophores, it is clear that the coördinating mechanism is nervous. Bethe (1895) showed a network of nerves to be present in the epithelium of *Cydippe*. Chun (1880) failed to find true nervous tissue in the ciliated furrows leading from the sensory region to the plate rows; but he believed this tissue to be nerve-like in transmitting impulses. Child (1933) found a clear picture of the nervous structure about the apical organ of *Pleurobrachia*, *Beroe*, *Bolinopsis*, and *Mnemiopsis* by staining them with a weak solution of methylene blue in sea water. He states (page 204), "There can be little doubt that these structures represent a real, morphologically differentiated nervous system." He failed, however, to find a definite structure, by staining, between the plates of *Pleurobrachia*. The stain showed granules in the outer body wall between the plates. The irregular arrangement of these granules could not indicate a definite transmitting structure. Child found definite structures between the plates of *Bolinopsis* and *Mnemiopsis* which he interpreted as nerve terminations. I have found these staining bodies in *Mnemiopsis,* but, although they are definite as granules (Fig. 2), I question any justification in assuming them to be nervous from their staining qualities alone, since methylene blue usually shows true nervous tissue to be cellular. This structure is in the epithelium above and independent of the food canals and is quite definite (Fig. 3). The granules were just as clear around the bases of the swimming plates as they were between these plates. Do these granular bodies around the plate bases represent nerves also? If so, these nerves are of a peculiar type since there are no indications of cells. I see no reason for calling either the interplate structures or the granular mass around the plate bases true nervous tissue, but some type of transmitting element is certainly present along the swimming plate rows as has been shown by physiological tests.

That the regulating center lies in the aboral zone is well established. In *Mnemiopsis,* usually the eight paddle plate rows are synchronized. At times when the animal is not moving and the rate of beating is decreased, a row of plates will beat independently of the others. Then we must assume that the regulating center in the aboral zone loses control with the lowering of the rate of beating. The coördinating mechanism in a single row is more definite physiologically than is the aboral center. In each row, the plates are synchronized perfectly even at the low speeds of movement. Even if a plate or two in a row be either cut out, held firmly, or cooled, the rhythm in a single row of *Pleurobrachia* is not interrupted (Parker, 1905*a*). According to Parker, the individual plate rows of *Pleurobrachia* are often independent of the others. The beating of the plates on the auricles is usually not synchronized with the beating of the other plate rows, nor do they beat at the same rate. Furthermore, the metachronal wave in the auricle is rarely continuous. Usually, this wave begins at the auricle tip, moving on one side to the adtentacular plate and on the other side to the oral lobe. These facts show further the localized nature of the transmitting and coördinating elements in *Mnemiopsis.*

The transmitting system, as indicated by the metachronal wave, in *Mnemiopsis* is polarized. Impulses proceed along the plate rows from the aboral zone to the oral zone. In the uninjured animal living in its natural environment, this transmission direction is never reversed. Reversal of the metachronal wave in *Pleurobrachia* occurs occasionally, but this could not be induced in *Mnemiopsis* (Parker, 1905*a*). Reversal of the effective beat does not occur in *Mnemiopsis* (Parker, 1905*a*:

Mayor, 1912), and it occurs only rarely in *Bolinopsis* (Child, 1933). Reversal of the effective beating of cilia of sea anemones is commonly known (Parker, 1905*b*, 1905*c*, 1917; Parker and Marks, 1928). Since ctenophores are related to the actinians, this led me to seek the evidence of paddle plate reversal in *Mnemiopsis.*

The excised pieces of *Mnemiopsis* previously described in this paper (Figs. 4, 5, and 6) certainly show a reversal of the effective paddle plate beat. It is mechanically impossible for the piece to reverse its direction of whirling without reversing also the effective paddle plate beating. The pieces used in this experiment remained on the one side while rotating. This reversed whirling followed any type of touching stimulus. Furthermore, the stimulus had the same effect whether it was applied anywhere on the jelly or directly on the paddle plate row. As far as could be observed, the aboral plate was the first to beat at the beginning of the normal metachronal wave and also at the beginning of the reversed effective beat. This gives proof to the dominance of the aboral region, which is in agreement with Child (1917). I did not see reversal of the metachronal wave of the paddle plates in the excised pieces of *Mnemiopsis.* Reversal of the metachronal wave in *Pleurobrachia* has been observed by Child (1933), who associated the lower degree of reversibility in the lobate forms with their higher degree of differentiation of the transmitting path.

When *Mnemiopsis* was cut transversely, dividing the animal into oral and aboral halves, the plates in the aboral half regained their rhythmic beating first. The fact that rhythm is slowly reëstablished in an oral half shows further the dominance of the aboral zone. Though the oral half established rhythmic beating within 24 hours, this was undoubtedly due to the reorganization of the tissue to reform the aboral center, since this center was completely reformed within 48 hours. Because of this rapid reformation of transmitting tissue, cutting a single plate from a row does not interrupt the rhythmic beating. This reorganization must have occurred in *Beroe* during the experiments of Eimer (1880), who observed the movements of the oral half to be indistinguishable from those of the aboral half. Lillie (1914), who cut strips of paddle plates from *Eucharis,* reported that they rounded up in much the same manner as did pieces of *Mnemiopsis* in my experiments. He stated that the two ends of the paddle plate row failed to fuse. This was true in pieces of plate rows of *Mnemiopsis* (Fig. 6). Lillie saw that the beating wave continued even over the open space in the jelly and concluded the impulse to be an electrical one which might well be transmitted across the gap through the water. A rapid reformation of the tissue in cut regions of *Mnemiopsis* demonstrates the possibility of the reformation of the transmitting structure around the gap. This, then, instead of electrical impulses passing through the water, accounts for the transmission of impulses across the gap in pieces of *Eucharis*.

SUMMARY

1. The apical organ in the aboral zone is the center from which impulses travel out into the plate rows.

2. The aboral impulses synchronize the plate movements in all eight rows except during slow movements.

3. At low rates of plate beating each row becomes independent.

4. The synchronism in the plate rows of a quadrant as a unit is more persistent than in the eight plate rows as a unit.

5. The highest type of synchronized unit is demonstrated by a single plate row.

6. A single plate row beating independently of other plate rows, whether normally located on the animal or detached from it, exhibits an aboral dominance.

7. Swimming plates do not reverse either their metachronal or effective beat while located naturally on the animal.

8. Swimming plates reverse their effective beat on isolated pieces of plate rows, but they do not reverse their metachronal wave.

9. Dilute solutions of methylene blue show the interplate structures in the plate rows to be entirely granular in nature without exhibiting any cellular elements.

10. The coördinating system of *Mnemiopsis* is demonstrated more definitely by physiological tests than by staining results. This system is superficially located along the plate rows. The fact that single isolated plates show a certain amount of autonomy and the fact that transmission in a plate row is immediately reëstablished following its interruption as produced by a cut show this system to be much more localized than has been heretofore believed.

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INDUCED OVULATION AND ARTIFICIAL FERTILIZATION IN THE FROG

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The relationship of the anterior pituitary to sexual maturity has been demonstrated by homoplastic implants in every major group of vertebrates. Various effects from precocious sexual maturity to induced ovulation have been reported for fishes (15); for Amphibia as represented by frogs (24, 25), toads (14, 16), *Triturus* (2, 20), *Triton* (2, 20); for reptiles as represented by snakes (15, 16); for birds by pigeons (6); and mammals by rats, mice, cats, rabbits, guinea pigs, and monkeys (6). The purpose of this paper is to summarize briefly the work on the Amphibia and to present considerable new evidence as to the quantitative factors involved and a dependable technique for induced ovulation and artificial fertilization in the frog, *Rana pipiens.*

In general, the extract of whole sheep or cattle anterior pituitary (8) has not proven to be effective with frogs and toads, though there are possible exceptions with *Rana vulgaris* (3) and *Discoglossus pictus* (17). Both the whole sheep pituitary extract and the Antuitrin-S from pregnant human urine, as dispensed by Parke, Davis and Company, have been useful in inducing ovulation with *Triturus* and *Ambystoma* (11, 22), though the Antuitrin-S seems to be more potent. Neither of these extracts has proven useful with toads (18).

Heteroplastic implants from the hen, rat, pig, dog, cattle, snake, fish, opossum and frog proved to be ineffective with respect to inducing ovulation in toads (3, 7, 15). This raised the question of "specificity," especially since the toad, *Bufo vulgaris,* reacts only to homoplastic implants while the toad pituitaries are effective when implanted in the frog, *Rana vulgaris* (3). Recent evidence (23) does not support this contentention of species specificity of pituitary, since with large doses of pituitary from the garpike, *Lepidosteus,* both the toad, *Bufo americanus,* and the frog, *Rana pipiens,* were induced to ovulate. It was suggested in this paper that previous failures may have been due to insufficient implantations. While heteroplastic implants between fishes and Amphibia may prove effective, no amount of pituitary from the albino rat (23) would induce ovulation in either frog or toad. Further evidence for the quantitative rather than qualitative relationship of the pituitary to ovulation is presented in this paper.

Heteroplastic implants among the Amphibia have proven to be effective in the following cases:

Donor	Recipient
Bufo vulgaris	*Rana vulgaris* (3)
Rana pipiens	*Triturus viridescens* (1)
Bufo terrestris	*Triturus viridescens* (4)
Rana pipiens	*Bufo americanus* (23)

Homoplastic implants, as a rule, prove more effective. The following cases of successfully induced ovulation as a result of homoplastic implants have been reported among the Amphibia:

Eurycea bislineata (20)
Bufo arenarum (16)
Bufo d'Orbignyi (16)
Bufo marinus (14)
Rana pipiens (24, 25)
Triturus viridescens (2, 20)
Triton cristatus (2, 20)
Steriochilus marginatum (20)

The normal breeding season for eighteen species of frogs and toads is reported by Wright (26).

MATERIALS AND METHODS

Eggs of *Rana pipiens* have been secured from three hours to four days after initiating pituitary treatment, from August 26 to June. In all probability this period can be further extended to include the summer months. Eggs and tadpoles of *Rana catesbiana* were secured during the summer.

Rana pipiens is obtained from Vermont after October 1, so that the normal hibernation supply of reserve food is stored within the body. The frogs are not crowded and are supplied with just enough water to keep the body moist. It is best to keep the animals in a cold room at about 10–12° C. until they are used. Strong solutions of potassium permanganate will prove useful in keeping down the red-leg which often develops. Forced feeding is not necessary, though this might facilitate the production of a second batch of eggs during a single season. Sudden changes in temperature should be avoided. At room temperature the reserve food material is apparently rapidly consumed and the eggs, even if layed, are not normal.

The pituitary is best secured by inserting the scissors into the mouth at the angle of the jaw and, with two cuts, severing the upper part of the head from the body. Care must be taken to avoid injury to the pituitary, which is situated just posterior to the level of the eyes. The head is then inverted and the floor of the brain case is removed by cutting anteriorly, on either side of the brain, as far forward as possible. By gently lifting the bony base of the brain case and inverting it, the small pink pituitary will be seen adherent to the " brain side " of this bony plate. Occasionally the pituitary may remain attached to the brain, surrounded by a flocculent white mass, where it can easily be picked off.

The pituitary may be used immediately or may be preserved in distilled water or in 10 per cent alcohol. Water suspensions have retained their potency for over two months when kept in the ice box at 4° C. The pituitary is macerated when fresh so as to make a homogeneous suspension. Whole pituitary injected through a large hypodermic gives good results. The extraction method (8) has not been tried since it has not been necessary.

Experiments to be reported here demonstrate that there is a quantitative rather than a qualitative difference between the male and the female pituitaries. For this reason the pituitaries from males and from females are segregated and are put up in concentrations which represent one female or two male pituitaries per cubic centimeter of suspension. This is, then, a suspension of known concentration which may have an unlimited life if kept in an ice box.

Injections of the pituitary suspension are made directly into the body cavity. Following injection with a small hypodermic the frog is left in a dry container for a short period so as to allow the incision to heal over and prevent escape of the suspension.

When amplexus is desired, control of egg-laying time is somewhat more difficult. Amplexus is unsuccessfully attempted when the male alone is injected, but is successful within twenty-four hours after proper injection of both male and female. Eggs will normally be laid during the early morning hours of the third day after two double injections of female pituitaries, giving rather high percentage of fertilization. There is greater percentage of fertilization, approximating 100 per cent, if the male is first injected twenty-four hours after the first injection of the female. This simply indicates a quicker reaction of the male, and amplexus is the necessary stimulus to the shedding of sperm.

In controlled fertilization experiments it is not necessary to inject the males, since macerated testes of untreated males in any season will give active sperm within 20–30 minutes after dilution. The optimum concentration is about two testes to 10–15 cc. of pond or spring water.

Fertilization will not take place in a suspension of frog sperm in amphibian Ringers. The spring water suspension of sperm will give normal fertilization as long as six hours after maceration of the testes, although the optimum time is approximately thirty minutes. In serial experiments where eggs are to be fertilized over a period of several hours one testis may be removed from each of two males and the pithed animals kept in the ice box until such time as the fresh sperm suspension is desired. The sperm suspension should be used at room temperature and should be spread thinly over the bottom of a finger bowl. The eggs can be stripped into this suspension, gently shaken (to separate), and fertilization will be at a maximum. Normally the eggs will undergo the second maturation division (which was interrupted at the metaphase to await insemination) within half an hour or so.

Stripping is most easily accomplished with least strain on the frog or injury to the eggs by grasping the body of the female in the right hand, legs extended between the thumb and forefinger, and with the back of the frog against the palm of the hand. The left hand can be used to displace the frog's legs and with gentle, even closure of the right hand, the pressure directed posteriorly, the eggs will slowly issue from the uterus. By this method the eggs may be stripped entirely at one time or in groups of fifty or more into each of 20–30 Stender dishes. Fertilization can best be accomplished as described above, by stripping directly into a concentrated sperm suspension which has been standing for thirty minutes. Attempts have been made to use an artificial salt solution which has the same osmotic pressure and salt balance as the oviduccal fluid of the frog (21) and should, therefore, not allow swelling of the jelly. This fluid does not prevent jelly swelling but does prevent fertilization. There may be some lethal action of the salt on sperm.

The eggs are flooded after about fifteen minutes, using the same water that was used to make the sperm suspension. In half an hour, after the jelly has swollen, the eggs should be freed from the bottom of the finger bowl. If the eggs are flooded too soon or a too dilute suspension of sperm is used, the jelly of the eggs will swell before any sperm can reach the cortex. Rotation of the eggs, the sign of successful fertilization, is first seen within an hour after the complete swelling of the jelly. Rotation in these cases of artificial insemination is not, however, a necessary sign of normal fertilization since the jelly is often adherent to the dish or to adjacent eggs, exerting enough pressure to prevent normal rotation. The eggs, which are surprisingly resistant to mechanical injury when covered with jelly, are cut apart with sharp scissors within a few hours.

The eggs will not remain in good condition in the uterus for more than a few hours unless the frog is kept in a cold room at 10° C. or lower. If the injection of pituitary suspension is properly timed in relation to placing the frog in the cold room, it is possible to slow up the passage of eggs from the ovary to the uterus. Care must be taken not to completely nullify the activity of the pituitary. Normally the eggs will accumulate in the uterus until they have all left the ovary unless they are forcibly removed by stripping or an insufficient quantity of pituitary has been injected. Ovulation can be induced in the cold room with stronger doses of pituitary. When the eggs are spontaneously laid by the female it is difficult, if not impossible, to fertilize them because of the mechanical impediment of the jelly. With proper control of temperature and pituitary a single pair of frogs should give healthy gametes over a period of two days at any time of the year.

It is possible, in the above manner, to secure a female in a condition rarely found in nature. A dissected female may show eggs in every step of the process: in the ovary, in the body cavity, entering the ostium, many in the oviducts, and accumulating in the uterus.

Experiments

A total of more than seventy-five sets of observations were made, twenty-six of which are reported above in composite form (Table I). The study included an analysis of the effect of the implantation as opposed to the injection method; the possible effect of the presence of the male and amplexus on ovulation; the relative potencies of male and female pituitaries in respect to both ovulation and amplexus; the stability of aqueous and alcoholic suspensions of frog pituitaries; the amount of pituitary needed in different seasons to induce ovulation; a few cases of the inter-specific effect of pituitary injection; and the possible use of mammalian extracts in respect to amphibian ovulation.

A few very recent observations (November, 1933) have suggested that the state of the female donor may have some relation to the potency of the pituitary. Mature female frogs (*R. pipiens*) were injected with whole (unmacerated) pituitaries taken from immature female frogs. Injection was through a fairly large hypodermic which would allow passage of the whole pituitary but would not involve sufficient injury to allow escape of fluid from the body cavity. In every instance ovulation occurred within twenty-four hours after a single injection of two such pituitaries. This quick reaction may have been caused by the special potency of the pituitaries rather than by the slight variation in injection technique. It is significant that two pituitaries are sufficient, if injected in the above manner, to induce ovulation in frogs within twenty-four hours during the fall months.

TABLE I

Ovulation in Rana pipiens with Pituitary

Exper. No.	Date	Purpose	Pituitary Source-Condition	Method	Total Pit.	Dose per day	Result	Conclusions
1010A	10–10	Determine dosage	♂ ♀—whole	Implantation	8	2–2–2–2	Eggs—5th day	Implantation method often effective but not entirely dependable. Ovulation in October.
1010B	10–10	" "	" "	"	12	2–2–2–2–2–2	No eggs	
1010C	10–10	" "	" "	"	6	2–2–2	Eggs—4th day	
1010D	10–10	Control	0	0	0	0	No eggs	
1105C	11–5	Concentrated dose	♂ ♀—whole	Implantation	8	4–0–4	Eggs—4th day	More concentrated dose tends to hasten ovulation.
1115A	11–15	" "	" "	"	8	4–4	Eggs—3rd day	
1126A ♂	11–26	Influence of the male	" "	"	4	2–2	Amplexus	Male reacts more quickly, hence eggs are not fertilized.
1126A ♀	11–26		" "	"	8	2–2–2–2	Eggs—unfert.	
1126B ♂	11–26	" " " "	" "	"	8	2–2–2–2	Amplexus—3rd day	Ovulation hastened by stronger dose. Eggs fertilized naturally.
1126B ♀	11–26	" " " "	" "	"	6	4–0–2	Eggs—fert.	
1126C ♂	11–26	" " " "	" "	"	0	0	No amplexus	No amplexus unless male is treated even in presence of ovulating female.
1126C ♀	11–26	" " " "	" "	"	8	2–2–2–2	Eggs—unfert.	
1206A ♂	12–6	" " " "	" "	"	8	2–2–2–2	No amplexus	Amplexus attempted frequently on 2nd and 3rd days: unsuccessful when female untreated.
1206A ♀	12–6	" " " "	" "	"	0	0	No eggs	
1206C ♂	12–6	" " " "	" "	"	4	0–2–2	Amplexus	Conditions best for natural fertilization when male treatment staggered.
1206C ♀	12–6	" " " "	" "	"	6	2–2–2	Eggs—100% fert.	
1214B	12–14	Sex potency	♀—whole	"	6	2–2–2	Many eggs	Female pituitaries relatively about twice as potent as male pituitaries. Two ♂ pituitaries per day representing a sub-minimal dose in respect to inducing ovulation.
1214C	12–14	" "	♂— "	"	12	2–2–2–2–2–2	No eggs	
1219D	12–19	" "	♀— "	"	6	3–3	Many eggs	
115B	1–15	" "	♂— "	"	15	3–3–3–3–3	Few eggs	
115C	1–15	" "	♂— "	"	12	4–4–4	Eggs	
126A	1–26	Injection method	♀—water suspension	Injection	4	2–2	Eggs	In January 2 double ♀ injections adequate.
126B	1–26	"	♂ " "	"	8	2–2–2–2	Few eggs	Male pituitaries not so potent as female.
126C	1–26	" "	♂ ♀— " "	"	6	2 ♀–4 ♂	Eggs	No antagonism between ♂ and ♀ pituitaries.
205B	2–5	" "	♂— " "	"	8	4–4	Eggs	Twice as many ♂ as ♀ pituitaries necessary.
414A	4–14	Stability suspension	♀— " "	"	2	2	Eggs	Suspension retains potency 2 months, ice box.
414C	4–14	" "	♀— " "	"	3	1–1–1	Few eggs	Ovulation easily induced nearer breeding season.
823A	8–23	Alcoholic suspension	♂—alc. susp.	"	15	5–5–5	Eggs	Ovulation can be induced in August.
823B	8–23	" "	♂— " "	"	9	3–3–3	No eggs	Strong doses needed in late summer.
23C	8–23	" "	♀— " "	"	6	2–2–2	No eggs	" " " " " "
81003A ♂	10–3	Stability alc. susp.	♂— " "	"	10	5–0–5	Amplexus	Alcoholic (10%) suspension stable for at least two months not in ice box.
81003A ♀	10–3	" " "	♂— " "	"	10	5–0–5	Eggs	

It is well known that the pituitary of castrate male rats is more potent than the pituitary of either normal male or normal female rats, and the pituitary of spayed female rats acquires additional potency over that of the normal female rat pituitary. Recent observations on the frog suggest that the pituitary of immature female frogs is at least as potent as that of mature female frogs and may be more potent in relation to induced ovulation out of season.

Summary and Conclusions

1. The injection of a suspension of macerated frog pituitary has proven to be more rapid, more consistent in its effect, and more economical in the number of pituitaries needed, than has the method of implantation. The injection method has never failed when mature females of *R. pipiens* have been injected with adequate doses of *R. pipiens* pituitary.

2. Ovulation induced in the manner described in this paper does not represent an all-or-none reaction. The number of eggs that leave the pituitary depend, at least in part, on the quantity of pituitary injected.

3. The pituitary may be macerated in distilled water and kept in the ice box for at least two months and will retain its potency, or it may be preserved in 10 per cent alcohol.

4. The season of the year need not be a barrier to ovulation provided sufficient pituitary suspension is injected. Eggs have been obtained from August 26 to June 2.

5. The male and female pituitaries are not equally potent. The female pituitary is at least twice as potent as that of the male in respect to inducing ovulation. Possible seasonal variations in pituitary potency have not been determined.

6. There is no sex specificity of the pituitary hormone. Alternate injections of male and female suspensions into a female will induce ovulation, or into a male will induce amplexus. Potency differences are quantitative.

7. Amplexus is unsuccessfully attempted when the male *alone* is injected with either male or female pituitary suspensions, and the female will react by ovulation with or without the presence of the male. Ovulation in the female will not induce the male to attempt amplexus unless the male is also injected, and the reaction is therefore not related to ovulation.

8. Amplexus and normal fertilization can easily be induced in the laboratory by the methods described in this paper. In almost any season two female pituitaries injected daily will result in ovulation on the

second to the fourth day. The reaction may be hastened by stronger doses of pituitary.

9. Ovulation has been induced in *Rana catesbiana* and *Rana palustris* by the use of pituitary suspension from *Rana pipiens*. The dose necessary to induce ovulation in *R. catesbiana* is somewhat larger than that necessary for either of the other two species.

10. Mammalian pituitary extracts, whole sheep pituitary, and the Antuitrin-S from pregnant human female urine have proven to be entirely ineffectual in respect to the ovulation in *Rana pipiens*.

11. The males need not be injected with pituitary since they will give functional sperm in spring water at any season of the year. The only purpose in injection is to induce amplexus.

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THE CONTROL OF THE DERMAL MELANOPHORES IN ELASMOBRANCH FISHES

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INTRODUCTION

The color changes in the elasmobranch fishes have received scant attention. Aside from the negative results recorded by Schaefer (1921) for *Raja clavata* and *Raja batis,* the only account of this subject known to us is that given by Lundstrom and Bard (1932) in a paper devoted to the action of the pituitary secretions on the color changes in the dogfish, *Mustelus canis.* In this paper it is shown in a very conclusive and adequate way that hypophysectomy is followed by a permanent paling of the fish and that this paling can be changed into a temporary darkening by the injection of pituitary extract. It is further shown by these authors that the changes thus experimentally induced agree in general with those normally excited in this fish by alterations in the tint of the background.

In their account of these changes Lundstrom and Bard give a very full description of the alterations of the fish from light to dark, but the reverse change is passed over with very slight comment. Although no specific statement is made by them on this point, it seems probable from the contexts in their paper that they regard the light phase of the dogfish as due to a decrease in the amount of the pituitary secretions in the fish's blood or possibly to a complete absence of these secretions from this fluid. According to them, this condition is in contrast to that in which there is an abundance of these secretions which thus induces the dark phase in the fish. That this dark phase is due to pituitary activity seems to us adequately and sufficiently shown and in this respect our own results fully agree with those of Lundstrom and Bard, but that the light phase is the consequence of the mere absence of the pituitary secretions appears to us quite unlikely as the observations recorded on the following pages tend to prove. Our own work was carried out on the smooth dogfish, *Mustelus canis,* partly at the Woods Hole Oceanographic Institution and partly at the Marine Biological Laboratory. To the Directors of these two establishments we wish to express our sincere thanks for the facilities and materials supplied us by which our investigations were made possible.

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[1] Contribution No. 36.

CHANGES IN THE GENERAL COLORATION OF THE DOGFISH

When a light-colored dogfish is placed in a black-walled tank illuminated from above, the fish quickly becomes dark. The time required for this change is from half an hour to an hour or two, and the subsequent retention of the fish in the dark-walled aquarium does not deepen further its tint.

Whether of light or dark shade in the beginning, dogfishes from which the eyes have been removed become quickly dark and remain indefinitely so irrespective of the shade of their surroundings. Such blinded fishes attain as a rule a deeper gray than the maximum shown by the normal fishes in a dark-walled tank. In fact, the darkest dogfish that we have ever seen was one whose eyes had been removed.

Dark dogfishes placed in an illuminated white-walled tank become gradually very light. Occasionally under such circumstances they fail to reach the extreme of light coloration and will maintain even in fully light surroundings a gray tint. This failure of full response has also been noticed by Miss Lundstrom, who called our attention to the frequent occurrence of cataract-like obscurities in the dioptric portion of the dogfish eye and suggested these defects as a possible explanation of this failure on the part of certain dogfishes to turn fully light. Unfortunately we were unable to test this suggestion. The majority of dogfishes, however, become extremely light in light surroundings and remain so. This paling is often associated with a pink tone that gives to the fish the appearance of a delicate blush. Such a coloration is doubtless brought about by the subdermal blood-vessels, which in consequence of the translucency of the skin in the light state show through this covering and thus affect the general tint of the fish.

The establishment of the light phase in the dogfish is a relatively slow process. A dark fish when put in a white-walled tank grows light at first rapidly and then very much more slowly till the maximum lightness is reached. This maximum is attained only after two or more days whereupon the fish remains indefinitely pinkish white.

What in general is thus slowly accomplished in a white-walled tank may be reached quickly by a subcutaneous injection of adrenalin chloride (Parke, Davis and Company). About five minutes after a dogfish has received a hypodermic injection of 0.5 cc. of diluted adrenalin, 1 part in 10,000, a general paling of the skin can be seen and half an hour later the fish is fully light. The degree of paling, even when 1 cc. of adrenalin is introduced into the fish, is seldom as pronounced as that seen in fishes from the white-walled tank, but occasionally the injected fishes reach what in our experience is the maximum of light coloration.

It is comparatively easy to show by placing the pectoral fin of a dogfish under the compound microscope that the dark phase of the fish is due to the expansion of the dermal melanophores and the light one to their contraction. Excellent figures of these conditions have already been published by Lundstrom and Bard (1932).

Fin Bands

If in a moderately dark dogfish deep cuts transverse to the rays of any fin are made or similar cuts are inflicted on the body, these cuts give rise to light bands or splotches which are clearly visible in the skin for many hours or even days thereafter. If the spinal cord of a dogfish is transected in the posterior part of the body, the portion of the fish behind the cut becomes irregularly slightly lighter. The light bands are best seen when they are produced on the pectoral fins and are most easily induced by making a well circumscribed cut completely through the fin from one face to the other at right angles to the rays. Such an incision is conveniently made by means of a chisel with a cutting edge of about one centimeter.

In the pectoral fin the course of the main blood-vessels can be easily seen through the translucent substance of that organ and the cut may be made either distal or proximal to the chief vessels, thus leaving the blood supply to the fin essentially undisturbed. The cut necessarily severs a number of the smaller vessels, but, if its position is well chosen, it can be shown to introduce no serious interference with the circulation. After such a cut has been made a light band quickly develops extending from the cut to the free edge of the fin. This band assumes the width of the cut, never more, and always extends over the fin distally from the cut, never proximally. It follows very closely the lines of the fin rays. Its detailed nature and fate depend upon the light or dark condition of the dogfish in which they are induced.

In a dark dogfish the band is a lightish area of irregular outline (Fig. 1). It is seen with certainty in from ten to fifteen minutes after the cut has been made and it reaches its maximum in about a day. After this it gradually fades out, to disappear completely in from two to three days. A narrow band three to four millimeters wide will, however, disappear in a little over a day; one of a centimeter wide will remain visible for as much as two to three days. The light band fades by being replaced with the dark coloration of the surrounding fin area, the center of the band near the periphery of the fin being the last to disappear (Fig. 2).

When a cut is made in the pectoral fin of an eyeless dogfish, a light band also appears in about a quarter of an hour and then follows the usual course of disappearance.

In a light dogfish the band is much more clearly defined in outline

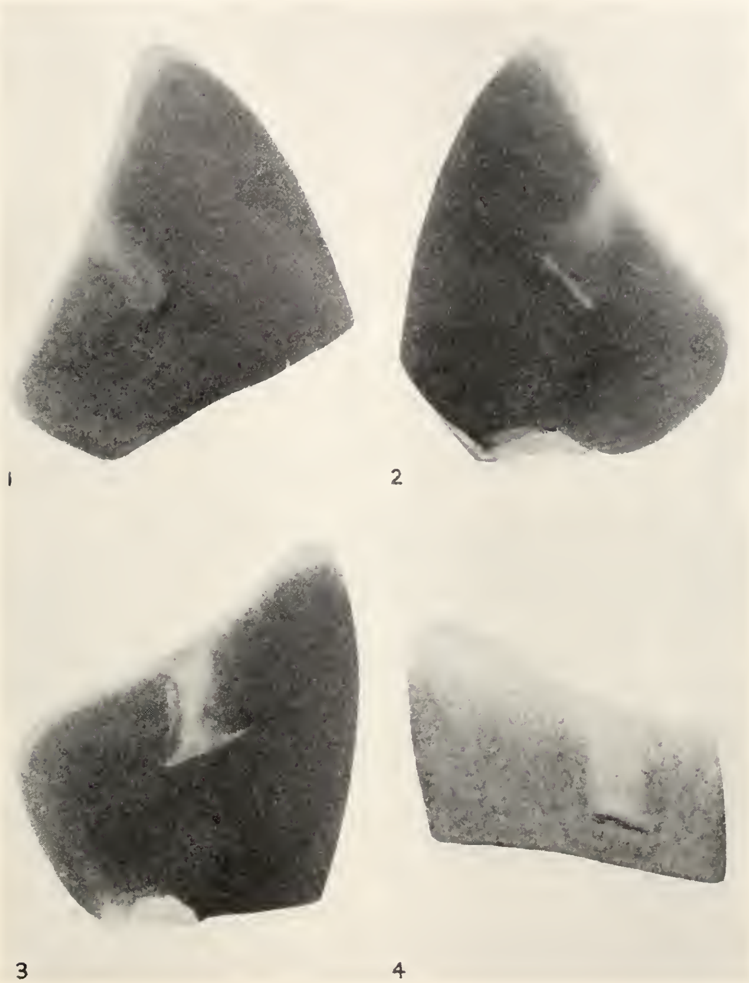

Plate I

Dorsal View of Pectoral Fins from the Dogfish, Mustelus canis

Fig. 1. A fin from a dark dogfish, showing a light band which appeared as a result of an incision transverse to the direction of the fin rays and about a centimeter and a half from the edge of the fin. The figure represents the state of the band about an hour after the cut had been made.

Fig. 2. A fin from a dark dogfish, showing a light band in process of gradual disappearance. The band was produced by the same kind of a cut as that shown in Fig. 1, but in the course of two days it became partly obliterated by the invasion of the general dark coloration.

Fig. 3. A fin from a dark dogfish, showing a light band in process of disappearance, an operation here locally checked by a longitudinal cut on one side of the band.

Fig. 4. A fin from a light dogfish, showing a light band several days after the initial transverse cut had been made.

than in a dark one. Its edges are sharply marked and they can be traced from the ends of the cut to the edge of the fin (Fig. 4). Although the fish may be extremely light in color, the band is always still lighter and agrees in tint with the white border of the fin. Unlike the light band on the dark fish, that on the light fish seems never to be obliterated. At least in all light dogfish kept by us in the laboratory tank the light band has persisted as long as the fish has lived, a period of at most about five days. When from a light dogfish with a light band the eyes are removed the fish darkens and the band eventually disappears as it does in an ordinary dark fish in which it had been induced by a cut.

Discussion

We are entirely in accord with Lundstrom and Bard in holding the opinion that the dark phase of the dogfish is due to the action of an expanding hormone carried to the melanophores from the pituitary body by the blood. We are convinced of the correctness of this opinion partly because of the facts advanced by Lundstrom and Bard and partly because of our own results. If the blood of a dark dogfish is drawn, defibrinated, and injected subdermally into light and into dark dogfishes, it will be found to have no effect upon the skin of the dark fishes but to produce a well-defined dark area on that of the light ones. This dark area, which may measure several centimeters in diameter, surrounds the point of injection and when examined under the microscope can be shown to have been produced by the expansion of melanophores.

Another observation that supports the general conclusion under consideration was made on operated fishes. If in a light dogfish a longitudinal cut parallel with the fin rays of the pectoral fin is made near the edge of a light band and the dogfish is put in a dark-walled tank, as the light band disappears it will last be seen next the longitudinal cut and on that side of it adjacent to the axis of the original band (Fig. 3). This method of disappearance is consistent with the idea of an invading, fluid-borne, expanding hormone such as has been claimed by Lundstrom and Bard.

For these and other reasons we are fully convinced that the dark phase of the dogfish results from the application of a blood-soluble, expanding hormone to the dermal melanophores.

The light-colored bands and their reactions as we have described them seem to us to be equally conclusive evidence that the light phase of the dogfish is dependent upon nervous action. To be sure, these bands follow the courses of the smaller blood-vessels and might therefore be attributed to an operative disturbance in the circulation. But, as already stated, the cut can be made either proximal or distal to the chief blood-vessels of the fin and the resulting band, which may easily be two or more centimeters long, is in large part so far from the exciting incision that the local circulation is completely undisturbed. This can be easily demonstrated by inspecting under a compound microscope the living pectoral fin of an operated dogfish. If the light ventral face of such a fin be examined, the flow of blood in the capillaries within the limits of the band can be seen to be as free and as abundant as it is in the regions outside the band. There is thus no reason to attribute the formation of the band to any circulatory irregularity, and the conclusion that is naturally arrived at is that the only other radial system, namely the nerves, is responsible for the bands.

The fin nerves run closely parallel to the fin rays and much more accurately so than the blood-vessels do. Since in light dogfishes the edges of the light bands follow very closely the courses of the rays, they must also follow the lines of the nerves. Hence we believe the light bands to be dependent upon the nerves. When a transverse cut is made in a fin a number of fin rays with their attendant nerves are severed and this severance is so vigorous a stimulus for these nerves that they excite the melanophores of their region to full contraction. The result of this response is that a light band is produced corresponding in extent to the area of distribution of the contracting nerves. Such a view coincides completely with what we know of the distribution of light spots and bands that result from skin cuts on other parts of the body of the dogfish. Spots or bands thus induced always extend from the region of the cut in the distal direction of the nerves and in no other. They are due in our opinion to the excessive activity of the severed contracting nerve fibers.

How long their contracting activity can be kept up we do not know. The bands in a light dogfish will maintain themselves essentially undiminished for as much as five days, the longest period that we have been able to keep an operated dogfish alive in the experimental tank. This period is much too short, judging from our results on *Fundulus* (Parker and Porter, 1933), to reach a condition of nerve quiescence and in our opinion the fin nerves concerned with melanophore contraction in the dogfish must be regarded as in more or less continuous action as a result of the irritation of the wound for a period of at least five days.

During this period, however, there appears to be in the dogfish a certain amount of decline in activity. This is seen in the disappearance of the light bands in dark dogfishes. As already pointed out, the light bands which are indefinitely persistent in light fishes disappear in at most two or three days in dark fishes. This disappearance we believe to be due to the action on the contracted melanophores of the expanding, blood-borne hormone which counteracts the contracting action of the nerves. When the fin nerves are first cut in a dark dogfish they are so vigorously stimulated that their activity predominates locally over that of the expanding pituitary hormone and the light band is formed. As time goes on, however, the melanophores of the band come more and more under the influence of the expanding hormone, a step rendered possible, we believe, by the subsidence in activity of the contracting nerve fibers. Thus the disappearance of the light band from the fin of a dark dogfish depends, in our opinion, in part at least on the gradual decline in the activity of the contracting fibers whereby the expanding hormone may assert itself. As already stated, we have never seen instances among dogfishes where this decline was complete, for if a dark dogfish in which the band has disappeared is put in a white-walled tank the band will reappear as the fish lightens. Nevertheless, we believe that in time this activity might completely subside, as it has been shown to do in the expanding fibers of *Fundulus* (Parker and Porter, 1933).

It must be clear from what has been stated that in our opinion the darkening of the dogfish skin is due to the action of an expanding blood-soluble hormone on the dermal melanophores of the animal. It must also be clear that the paling of the dogfish skin results from the action of contracting nerve fibers on the melanophores, an action which when excessive may overcome that of the expanding mechanism. Do the contracting nerve-fibers produce a contracting substance or neurohumor comparable to the pituitary expanding hormone? Thus far we must answer this question in the negative and in consequence of two lines of evidence. First, when the defibrinated blood of a light dogfish is injected into a dark one, the skin of the dark individual does not lighten, showing that such blood carries no dissolved contracting neurohumor such as is stated by Meyer (1931) to occur in certain flatfishes. Secondly, if the width of a newly-produced band in the pectoral fin of a light dogfish is accurately measured and if this measurement is compared with a second one of the same band in the same place made three days later, no increase in width can be noticed. This result is opposed to the view that the light band is due to a water-soluble neurohumor produced by the contracting nerve fibers and more or less like the expanding blood-borne hormone. If such a substance were present it most certainly would induce an expansion of the band. The evidence here presented, however, does not preclude the possibility of a locally produced contracting neurohumor insoluble in blood or lymph, but it makes clear that if such a neurohumor is present, it is probably lipoid-soluble and would therefore be essentially local in action.

The general conclusion to be drawn from this discussion is that the color changes in the dogfish are of two types, one from light to dark due to an expanding, pituitary hormone carried from the gland to the melanophores by means of the blood and other body fluids, and another from dark to light due to a contracting local action of nerves. It is interesting to note that of the many animals in which the cutting of nerves produces a change in coloration, the dogfish is the first one to be recorded in which this change is a paling. All other forms exhibit a darkening when their chromatophoral nerves are severed. This we think is due to the fact that in the dogfish, unlike other fishes, probably only contracting nerves are present and that consequently in this fish these nerves have no others with which to compete as the contracting nerves of other fishes apparently have. On stimulation these nerves in the dogfish therefore assert themselves without hindrance. The dogfish is further peculiar in that it presents a melanophore system in which the expanding action is unihormonal, modeled after that of an amphibian, and the contracting action nervous like that in most other fishes.

SUMMARY

1. The common dogfish, *Mustelus canis*, has a dark phase and a light one due to the expansion and the contraction of its dermal melanophores.

2. The dark phase is induced by pituitary secretions carried from the pituitary gland to the melanophores by the blood and lymph as described by Lundstrom and Bard.

3. The light phase is induced through the action of contracting nerves, and is not merely the result of the absence of pituitary secretions.

4. The expanding hormone is water-soluble. The contracting one, if there be such, is apparently not water-soluble.

5. The dogfish is remarkable as the first form to be described in which the cutting of nerves induces a contraction of the melanophores and a consequent lightening of the skin.

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STUDIES ON AMPHIBIAN METAMORPHOSIS

XIII. The Stability and Growth of Anuran Tympanic Membrane Following Larval Involution

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The differentiation and growth of the anuran tympanic membrane during the metamorphosis of the tadpole has until recently been studied only from the structural standpoint. Thus, Moldenhauer (1878) figured and described the histological structure of the tympanic membrane in *Rana esculenta* and *Rana temporaria*. More recently, Tokura (1925) worked out the peculiar fibrous complex typical of the dermal portion of the membrane in *Rana nigromaculata*, a variety of *Rana esculenta*. The writer (1928), working with *Rana pipiens*, was able to confirm the structural findings of the earlier workers and in addition determine the developmental interrelationship between the annular tympanic cartilage and the integumentary portion of the tympanic membrane and later (1931), the relationship of the columella to the development of the lamina propria portion of the membrane. The latter results may be briefly stated as follows: Any integument transplanted over the annular tympanic cartilage will develop into tympanic membrane[1] during larval involution. Conversely, integument of the tympanic membrane region fails to transform into tympanic membrane during metamorphosis when previously transplanted to the back or side. Extirpation of the annular tympanic cartilage inhibits the development of tympanic membrane, while transplantation of the annular tympanic cartilage beneath integument of the back or side initiates tympanic membrane formation in these tissues. In a like manner, extirpation and transplantation experiments with the columella were conclusive in showing that the latter structure is responsible for the development of the yellow fibrous portion of the lamina propria layer of the membrane.

The tympanic membrane as it is found at the close of larval involution appears to be fully differentiated both as regards external appearance and histological structure. Following larval involution, however, the membrane continues to increase in size and in fact does not attain its maximum dimensions until growth of the frog as a whole is complete, years later. This fact is provocative of several interesting problems which may be stated in the form of the following questions: Does the annular tympanic cartilage continue to exert a developmental influence on the growth of the tympanic membrane after larval involution is complete, or is the membrane's post-larval growth a self-sustaining phenomenon unrelated to the cartilage? Furthermore, is the histological integrity of the tympanic membrane at any stage of its growth dependent on influences arising from the annular tympanic cartilage or can it maintain its cytological structure when transplanted away from the cartilage?

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[1] The term *tympanic membrane* or *membrane* as used here and throughout the present paper refers only to the integumentary portion of the membrane and is not meant to include the lamina propria or internal epithelial layers.

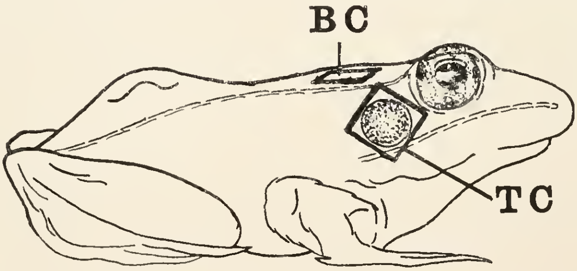
The results of the present paper concern experiments devised to answer the above questions. Briefly, they consist of the reciprocal transplantation of fully-differentiated tympanic membrane with integument from the back of newly-metamorphosed frogs. The subsequent development of tympanic membrane structures in the back-skin transplants would lead one to conclude that the annular tympanic cartilage is still productive of developmental influences following larval involution, while failure to obtain membrane development would lead to the reverse conclusion. Likewise, the maintenance of tympanic membrane histological structure, following transplantation to the back, would indicate post-larval stability and independence of the membrane, while cytological regression of the membranes would imply their continued dependency on the annular tympanic cartilage.

Materials and Methods

The species selected for the various transplantations were *Rana palustris* and *Rana clamitans*. Specimens of both these species were collected in considerable quantity in the vicinity of Lake Winnisquam, New Hampshire, during the months of July and August, 1932. Only individuals nearing the close of the involution period were collected. These were isolated in individual aquaria, thus making possible the selection for operative work of specimens whose exact post-metamorphic age was known.

The technique employed for the reciprocal transplantation of the tympanic membrane with integument of the back was as follows: Young frogs were first selected at a stage two to three weeks following the disappearance of all vestiges of a tail stump. At this time the tympanic membrane is fully formed, as regards external shape, coloration, and histological structure. The animals were now anesthetized in chloretone solution and under a binocular microscope membranous and integumentary squares of tissues removed for transplantation. In removing the tympanic membrane, a four-sided incision was first made in the skin of the neck region (*TC*, text figure 1) inclosing the membrane. The square of integument and tympanic membrane was now carefully loosened from the underlying musculature and annular tympanic cartilage by means of a small flat spear-head needle and the whole tissue finally stripped off by gently pulling with a pair of forceps. Considerable care is necessary in order not to injure the under layers of the tympanic membrane or the thin, delicate lamina propria covering the annular tympanic cartilage. A square of integument of approximately the same size was next removed from the middle of the back of the same animal (*BC*, text figure 1). Back integument is easily stripped off due to the relatively few points of adhesion with the musculature of that region. The square of back integument was now trimmed to fit the wound area left by the removal of the tympanic membrane and finally transplanted to that area. The tympanic membrane square of integument was next transplanted to the wound area on the back and the animal partially submerged in water so as to expose the two wound areas to the air. Within ten to fifteen minutes, the transplants had adhered sufficiently to permit total submergence of the animal.

The various animals so operated on were maintained in individual glass aquaria for the purpose of daily observation of the transplants and the appearance of macroscopic changes indicative of tympanic membrane development or regression, as the case might be. During this period it was not found necessary to feed the animals. When it later became evident that a specimen was destined to die soon, the entire animal was fixed in Bouin's fixative. The fixation of the transplants while still on the hosts was found desirable in that integumentary transplants removed from their host before fixation usually tend to curl up or wrinkle, making subsequent sectioning and histological examination difficult.



Results

Forty-seven reciprocal transplantations were made as described above using *Rana palustris* frogs and thirty-five using young *Rana clamitans* frogs. Of these, twenty-six of the former species and nineteen of the latter lived for at least three weeks following the transplantation of the grafts. Individuals dying before the three-week stage were not preserved or fixed for future examination, since it was believed that twenty-one days was approximately the minimum time necessary for development or regression of tympanic membrane structure under the conditions of the experiment. Only the results of individuals living for three weeks or more, therefore, are described below.

Development of Tympanic Membrane in Back Integument

Rana palustris transplants.—Of the twenty-six animals surviving the three-week period, fourteen were fixed within one to two weeks later, seven within two to three weeks later, while one individual lived for over eight weeks following transplantation. All individuals gave evidence of some degree of tympanic membrane formation as based on external appearance. Usually, however, only slight macroscopic changes were observed. These consisted mainly of coloration changes in the integument resembling the brownish hue of normal membranes. The characteristic iridescence of fully-formed tympanic membranes was usually not attained, however. Partial to complete " outlining " of the membrane was usually exhibited. Such " outlining " always occurred as a narrow, pale line corresponding to the outer rim of the annular tympanic cartilage beneath the integumentary transplant. In general, it may be said that the degree of macroscopic changes observed was correlated with the length of time the transplant was associated with the annular tympanic cartilage. Thus several of the older cases, including most of those living for from five to six weeks and the one surviving eight weeks following integumentary transplantation, developed quite typical tympanic membranes closely paralleling the normal membranes on the left side of the same individuals.

Histological sections gave evidence that definite characteristic cytological changes had occurred typical of tympanic membrane formation. These were also closely correlated in degree with the extent of the macroscopic developments recorded at the time of fixation of the transplants. The majority of the transplants exhibited but early histological signs of membrane formation. These included loss of the convoluted appearance of the stratum compactum, the partial disarrangement of the fibril bundles of the latter, and a diminution in thickness of the layer. The stratum spongiosum was also usually greatly reduced in thickness, while the cutaneous glands of this region were also reduced both in size and number. The transplants which had developed more complete macroscopic signs of membrane formation were characterized by a reëstablishment of the stratum compactum elements in slight wavy bundles characteristic of fully-formed tympanic membrane structure.

The stratum spongiosum was also quite typical in that cutaneous glands were either entirely absent or represented by small degenerating vestiges. Figure 2, Plate I, represents a section through one of the fully-developed membranes in which the above histological changes had occurred. The histological appearance of back integument of *Rana palustris,* from which this membrane was developed, is represented in Fig. 1, Plate I. The stage of development, previously described by the writer (1928) in the development of *Rana pipiens* tympanic membrane in which the basal cells of the epidermis undergo hypertrophy, was not found in sections of either *Rana palustris* or *Rana clamitans* membranes. Although this developmental stage may not be characteristic of the two latter species, it seems more logical to assume that due to the relatively short time interval occupied by this transitory hypertrophy (as found in *Rana pipiens*), the developing membranes as studied in the present work were not fixed at the exact stage necessary to show this effect.

Rana clamitans transplants.—Of the nineteen animals surviving the three-week period, the majority were fixed within one to two weeks later, four within two to three weeks later, while one lived for over seven weeks and another for over nine weeks following transplantation. The results of the *Rana clamitans* series were quite similar to those as described for *Rana palustris.* External signs of membrane formation again exhibited considerable variation in degree of development correlated closely with

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Explanation of Figures

1. Normal *Rana palustris* back integument at stage used for transplantation over the annular tympanic cartilage. Note the characteristic convolutions of the stratum compactum and the presence of numerous cutaneous glands. *Rana clamitans* back integument, as used for transplantation, presented practically the same histological appearance.

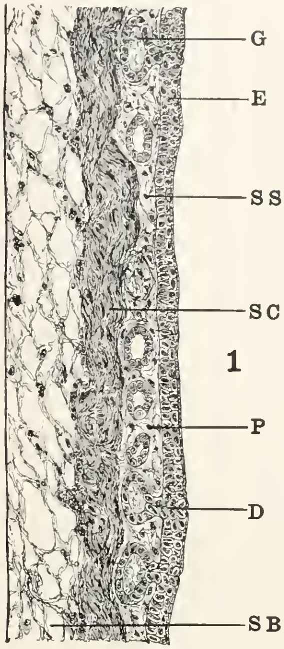
2. Fully-developed tympanic membrane formed in *Rana palustris* back integument transplanted six weeks before over the annular tympanic cartilage. Note absence of cutaneous glands and wavy appearance of stratum compactum.

3. Showing stability of fully-differentiated *Rana clamitans* tympanic membrane thirty-six days following autoplastic transplantation to the back. Tympanic membrane shown below region X; adjacent back integument shown above region X. The tympanic membrane has retained practically the same histological appearance that it had when transplanted.

Abbreviations

ATC, annular tympanic cartilage
C, tip of columella
D, duct of cutaneous gland
E, epidermis
EF, thickened elastic fiber region of lamina propria
G, cutaneous gland
IE, inner epithelial layer
LP, lamina propria
M, muscle
P, pigment cell
SB, subcutaneous connective tissue
SC, stratum compactum
SS, stratum spongiosum
TC, tympanic cavity
TT, tensor tympani
X, fusion region of transplant and adjacent back integument

PLATE I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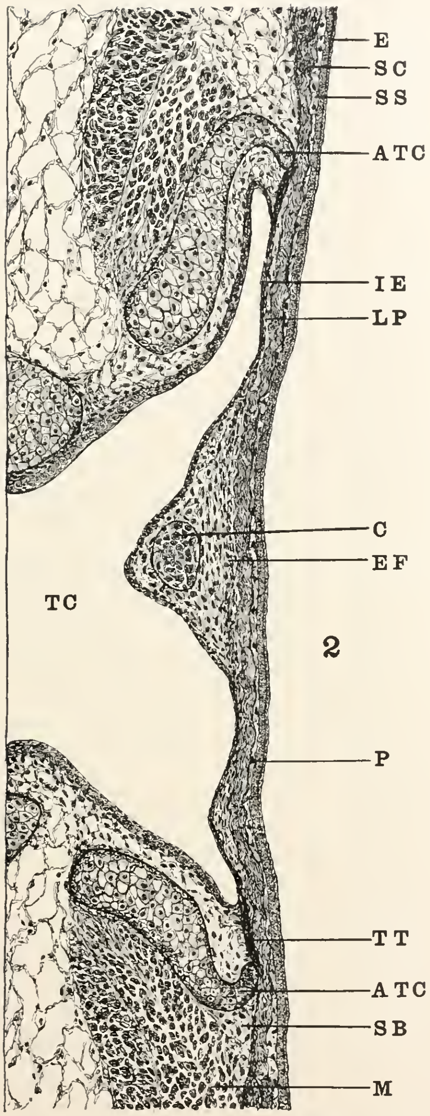
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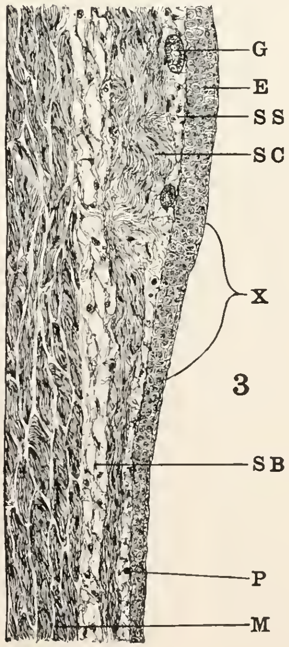
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the time the integumentary transplant was in contact with the annular tympanic cartilage. Coloration changes were somewhat different for the *Rana clamitans* series but characteristic of the species, while "outlining" of the area to develop into tympanic membrane was usually not as complete or distinct as compared with the *Rana palustris* series. The microscopic appearance of the developing membranes also closely approximated the conditions as described for similar developing membranes in *Rana palustris* integumentary transplants. Where differences occurred, they were concerned entirely with size variations of the integumentary layers and their cytological constituents. The various histological changes were always, however, characteristic of tympanic membrane formation. The two integumentary grafts maintained for seven and nine weeks, respectively, over the annular tympanic cartilage developed especially well-developed tympanic membranes both as regards external and microscopic appearance.

Stability of Tympanic Membrane following Transplantation

The behavior of the *Rana palustris* and *Rana clamitans* tympanic membrane grafts transplanted to the back was practically the same, for which reason they will be described collectively. When originally transplanted, the squares of integument containing the centrally located circular tympanic membranes were at first surrounded by a wound area separating them from the integument of the back. This wound area was shortly covered with epidermis, while formation of the subepidermal layers required a much longer period of time. The preponderance of grafts maintained their healthy appearance and became firmly attached to the musculature of the back. In fact, the attachment was usually more firm than that of the normal back integument. Several *Rana palustris* transplants, however, underwent degeneration resulting in partial disintegration. These were discarded.

The majority of the transplants underwent slight regressive changes as viewed externally. A partial loss of the characteristic tympanic membrane coloration occurred, although a distinctive brownish hue was always retained. The indentation of the membranes, quite characteristic when in their normal orientation over the annular tympanic cartilage, usually became progressively less pronounced. The above two macroscopic changes were always most evident in membranes remaining the longest on the back prior to the fixation period. In many cases where the transplants were fixed within four weeks following their transplantation, no macroscopic changes developed; the membranes appearing practically the same as when transplanted.

The microscopic changes observed in membranes exhibiting external regression were correspondingly slight in extent. Where they occurred at all, they consisted of but moderate thickening of the stratum compactum and stratum spongiosum layers. No cutaneous glands were ever observed developing in the latter layer, however, while the sub-epidermal pigment cells always appeared quite typical of tympanic membrane structure as regards size, number, and distribution. Figure 3, Plate I, represents a *Rana clamitans* tympanic membrane transplant thirty-six days following transplantation, which retained practically the same histological features it had when originally transplanted to the back. The results in general, therefore, show that tympanic membrane is subject to but slight regressive changes when transplanted away from the annular tympanic cartilage, while many cases exhibit no regression at all.

Discussion

The comparative slowness of tympanic membrane formation in back integumentary grafts transplanted over the annular tympanic cartilage calls for explanation. In this regard it may be said that the average time taken for the development of early tympanic membrane changes was approximately three weeks for either *Rana palustris* or *Rana clamitans.* The writer (1928) found, however, that similar developments in *Rana pipiens,* using the same type of transplantation, occurred on the average in about nine days. The comparative slowness in *Rana clamitans* as contrasted with *Rana pipiens* might be explained by the fact that the former species requires a considerably longer time for larval involution and hence development of its adult structures. This explanation, however, cannot be applied in the case of *Rana palustris,* since the latter species metamorphoses in about the same time as *Rana pipiens.* It seems more probable to attribute slowness in membrane formation to the fact that the transplanted back integument of the young *Rana palustris* and *R. clamitans* frogs was thicker and possibly less responsible to developmental changes as compared with the *larval* back integument used in the *Rana pipiens* transplantations. Furthermore, development was no doubt retarded due to the fact that the annular tympanic cartilage of young frogs, as used in the present work, possesses a less intense developmental influence as compared with the developing cartilage which initiated tympanic membrane formation in the *Rana pipiens* experiments.

The results of the present paper indicate that the structure of anuran tympanic membrane, following larval metamorphosis, is quite stable and not subject to any great degree of regression when contact with the annular tympanic cartilage is terminated. This stability of structure is apparently only true of the fully-differentiated membrane, since earlier work by the writer (1928) has shown that membranes in various developmental stages during larval involution tend to undergo regression back to normal integument following extirpation of the annular tympanic cartilage. It would appear, therefore, that the more fully-differentiated the tympanic membrane, the more stable it becomes and the less dependent it is on the annular tympanic cartilage in this respect.

The present work has shown that the annular tympanic cartilage of the young frog is still capable of inducing tympanic membrane formation in integumentary transplants placed over it. The intensity of this influence is not as great as has been shown to be true of the same cartilage in its developmental period during late larval involution, but the influence as such still persists. The question naturally arises, therefore, as to whether or not the annular tympanic cartilage actually operates to bring about the enlargement of the tympanic membrane as it occurs during the growth of the young frog. That the annular tympanic cartilage is wholly responsible for the enlargement of the tympanic membrane, seems evident from unpublished results of the writer which indicate that no increase in size of the membrane is possible following extirpation of the cartilage in young frogs. The problem therefore resolves itself into the question of just how the tympanic membrane increases its area under the influence of the annular tympanic cartilage. Two possibilities are evident in view of the present results. The enlargement of the membrane is due either to multiplication and growth of the various constituent layers already formed, or else to the transformation of peripheral integument into new tympanic membrane. Whether either one or both of the above methods operate to bring about membrane enlargement cannot be stated at this time due to lack of suitable data. In any case, the annular tympanic cartilage supplies the developmental or growth influences operative to bring about the enlargement of the membrane. The writer is more inclined to believe that the annular tympanic cartilage functions to transform peripheral integument into more tympanic membrane which is added to the more centrally located membrane already formed. While the results of the present paper strongly suggest this mode of membrane enlargement, the problem still awaits an adequate solution. Further experiments are planned regarding this question.

SUMMARY AND CONCLUSIONS

Previous work had shown that contact of the annular tympanic cartilage with overlying integument induced transformation of the latter into tympanic membrane during metamorphosis. Whether or not this influence persists following metamorphosis and whether or not maintenance of tympanic membrane structure is dependent on continued association with the cartilage was determined as follows:

Young *R. palustris* frogs were selected two to three weeks following complete metamorphosis. Integument including the tympanic membrane was removed and a similar piece of integument was cut from the back. Reciprocal transplantation of the two grafts was then made, autoplastically. Within three to five weeks definite though slight external signs of tympanic membrane formation were evident in the back-skin grafts over the annular tympanic cartilage. Histological examination showed slight to pronounced signs of membrane development. The membrane grafts made to the back showed only slight signs of regression, either macroscopic or histological.

Similar reciprocal transplantations were made on young *R. clamitans* frogs with somewhat similar results. Again only slight regression was observed in membranes transplanted to the back, while membrane formation in grafts over the annular tympanic cartilage varied from slight to very pronounced transformation.

The conclusion is reached that annular tympanic cartilage continues to exert its developmental influence on membrane formation for some time following metamorphosis, but with decreasing intensity. Also, that fully-formed membrane is quite stable and not susceptible to serious regression when removed from contact with the annular tympanic cartilage.

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THE AIR TURBINE FOR HIGH SPEED CENTRIFUGING OF BIOLOGICAL MATERIAL, TOGETHER WITH SOME OBSERVATIONS ON CENTRIFUGED EGGS

E. NEWTON HARVEY

(From the Loomis Laboratory, Tuxedo Park, New York, the Physiological Laboratory, Princeton University, and the Marine Biological Laboratory, Woods Hole)

There are two main difficulties to be encountered in centrifuging living cells at high speed. First, they may be crushed against the bottom of the container by the high forces developed. This can be obviated by suspending the material in a medium of graded density so that it comes to lie in a stratum of equal density and is completely cushioned against crushing. For this purpose mixtures of isotonic sucrose or raffinose [1] and salt solution may be used with material normally bathed in sea water or salt solution, while neutralized gum arabic will serve for fresh water forms.

Second, heating of material is bound to occur due to air friction unless the centrifuge is run in a vacuum or in hydrogen at low pressure, a procedure which introduces complicated accessory apparatus and is decidedly objectionable from the biological standpoint.

This difficulty is avoided by the use of the air turbine (Henriot and Huguenard 1925, 1927) perfected by Beams (1930, 1931, 1933) and fully described by him. Suffice it to say that a small top-shaped bearingless rotor (Fig. 1) with thirty grooves or flutings cut in its sides revolves on an air cushion of whirling jets of air, which hold it in place by the principle of Bernouilli. The constantly expanding air maintains the rotor a few degrees below room temperature. There are eight jets of air in the stator [2] I have been using, coming from diagonally bored holes of $\frac{1}{32}$ inch diameter which connect with a reservoir of air at a pressure that can be varied from 0 to 120 lbs. per sq. in. (Figs. 2 and 3). A vertical hole of $\frac{1}{12}$ inch diameter in the bottom of the stator is connected with an air supply at lower pressure and serves as a supporting jet. By the adjustment of the rotating air pressure and the supporting air pressure, rotors of varying size and shape can be made to revolve smoothly. A more recent design (Beams, Weed, and Pickels, 1933) of the stator omits the supporting pressure. The vertical hole is open to the atmosphere and the air sucked in automatically maintains the rotor in a stable position of rotation. Garman (1933) has described details of construction for efficient rotation.

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[1] A trisaccharide with higher molecular weight and greater density for isosmotic concentrations.

[2] Kindly constructed for me by Mr. A. J. Weed under Dr. Beams' direction.

Since eight holes of $\frac{1}{32}$ inch will deliver 1.61 cu. ft. of air at 100 lbs. pressure and one hole of $\frac{1}{12}$ inch diameter will deliver 3 cu. ft. of air at 10 lbs. pressure, the total air capacity necessary is about 18.7 cu. ft. per minute at 100 lbs. pressure. The air compressor should be capable of delivering this and should be a 5 H.P. outfit.

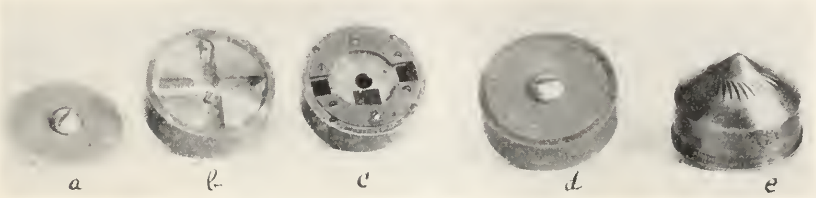

FIG. 1. Rotors for air turbine. (*a*) cover for tube type; (*b*) tube type rotor showing grooves for centrifuge tubes; (*c*) rotor for microscope observation; (*d*) tube type rotor with cover on; (*e*) bottom of rotor showing flutings.

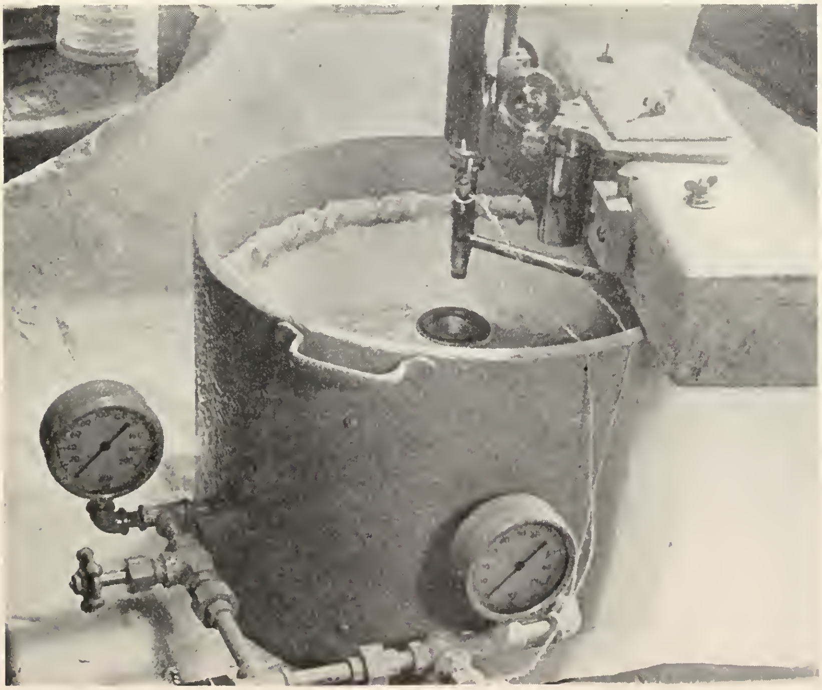

FIG. 2. Stator mounted in a steel protecting case with pressure gauges to read rotating and supporting pressures and microscope mounted for observing material. Note canvas for catching rotor when it is to be stopped or if it jumps out of stator.

The centrifugal forces developed at high pressures are enormous and adequate protection in the form of steel casing or sand-filled barriers must be provided to prevent injury from exploding rotors. A strip of canvas under the stator (Fig. 2) serves to catch the rotor if it becomes unstable and moves out of the stator.

Rotors of various types can be used. Beams (1931) has described rotors into which material can be introduced and from which it can be taken while rotating and also methods of observing the sedimentation velocity of particles (Beams, Weed, and Pickels, 1933). I have already described (Harvey, 1932) a type[2] designed for observation of material with the microscope while being centrifuged. The complete outfit is shown in Fig. 3 with rotor resting on stator, microscope, and illumination. The optical system is shown in Fig. 4. The light is a small 2-volt electric flashlight bulb, an image of whose straight filament is thrown by a lens on the cells to be observed, parallel to a radius of rotation. The chamber to hold the cells is made from Pyrex tubing, sealed and flattened at one end, and ground and polished to fit in position in the rotor. The straight end of the chamber where the cells are thrown by centrifugal force must come in position over the stellite mirror so that its image is reflected into the tube of the microscope. A perfect

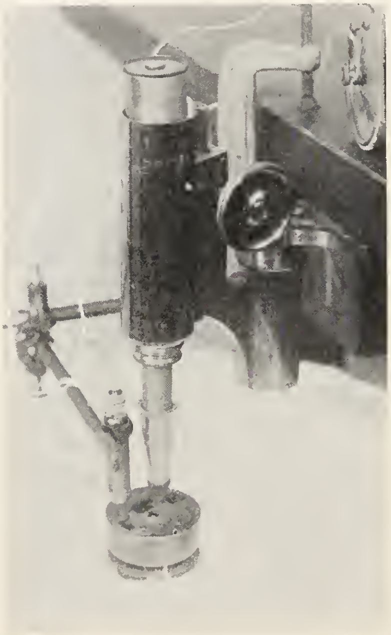

FIG. 3. Near view of rotor on stator with microscope and illumination.

image is obtained. Careful construction of the chamber and a cushion of picene will prevent breakage at relatively high centrifugal forces, but special treatment of the glass (Beams, Weed, and Pickels, 1933) is necessary at very high speeds. The chamber is shown in Fig. 5, *A*.

For centrifuging in tubes the rotor[3] shown in Fig. 1 is most convenient. This rotor can be made of solid steel rod 1¾ inch diameter, turned to shape, with two crossed grooves to hold centrifuge tubes milled in the upper surface. A cover of duraluminum fits over the grooves and is held in place by a machine screw. For low speeds the tubes can be made of thick-walled Pyrex tubing, but for high speed they are best made of duraluminum or aluminum, which are light and do not easily corrode. Holes are bored in square duraluminum rod and the end rounded in one plane to fit the groove in the rotor as shown in Fig. 5, *B*. Only ordinary precautions are necessary to make tubes of the same weight, since the rotor assumes its own position of equilibrium when revolving and a considerable amount of unbalance does not interfere with the stability of the rotor, provided the supporting

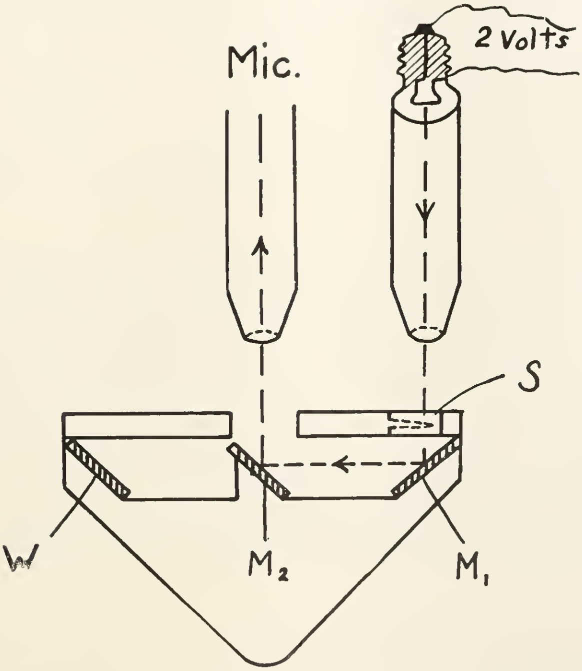

Fig. 4. Optical system for microscope observation of material rotating at high speed.

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[3] Constructed by Mr. P. Miller at the Loomis Laboratory, Tuxedo Park.

and rotating pressures are properly adjusted. The rotor is started by placing it on the stator and turning on the air. It is stopped by scraping it off the stator onto the canvas with a strip of cardboard while rotating.

I find that sensitive organisms like *Paramoccium* live for days in culture medium containing duraluminum or aluminum filings but are killed in twenty-four hours in contact with monel metal filings. *Arbacia* eggs develop into plutei in contact with duraluminum filings.

The rate of rotation can be determined in a number of different ways, most simply by a stroboscopic method. A mark of black paint is made at one point on the rotor and this is observed with a Neon lamp [4] flashing at a rate that can be varied. This rate is obtained by setting a number of brass contact points in a bakelite commutator disk in concentric circles of say 4, 6, 8, 10, 12, 15, and 18 contacts. The disk is revolved by a variable speed motor whose speed can be read on a tachometer. A contact spring presses against the contact points on the disk, thereby applying 180 volts from B batteries to the Neon lamp. The speed of the motor is adjusted and a contact circle is selected so that the Neon lamp flashes at the same rate as the rotor revolves, when the mark of black paint will appear to stand still. The revolutions of the motor times the number of contact points gives the speed of the rotor. It is necessary to make certain that the speed of the rotor is not some multiple of the stroboscopic rate. This can be ascertained by flashing the Neon lamp at a relatively low rate and observing the sequences of images of the black mark on the rotor as its speed increases. Three images mean ⅓ the speed of the flashing Neon lamp, two images ½ the speed, one image the same speed. When the speed of the rotor is increased to the point where a second single image appears (the Neon

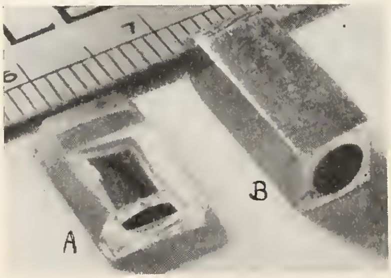

FIG. 5. Glass chamber (*A*) for microscope observation and duraluminum tube (*B*) for high speed centrifuging. The scale shows dimensions in mm.

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[4] The Neon lamps used in television work with a large rectangular glowing electrode give enough light for use in a slightly darkened room.

lamp flashing at the same rate), its speed is twice that of the Neon lamp. In this way a curve can be plotted relating speed of the rotor to rotating pressure.

This curve can be checked in another way. The top of the rotor is painted flat black except for a small area of metal which is polished. An intense beam of parallel light is directed on the rotor so that the polished area reflects the light into a photocell. The photocell current is amplified and thrown into a loud speaker and the pitch of the sound compared with a tuning fork of known frequency. Or, an electrically driven tuning fork can be simultaneously connected with the photocell amplifier and the beats observed.

In this manner the steel rotor used for duraluminum tubes was found to have a speed of 800 R.P.S. at 20 lbs., 1,140 R.P.S. at 40 lbs., 1,460 R.P.S. at 60 lbs., and 1,800 R.P.S. at 80 lbs. rotating pressure. Since the end of the tubes are 1.5 cm. from the center of rotation the centrifugal force at 20, 40, 60, and 80 lbs. pressure is, respectively, 38,000, 78,000, 128,000, and 144,000 times gravity.

Using the tubes and egg cells suspended in isotonic sugar plus sea water mixtures of graded density, it is an easy matter in a very short time to stratify eggs which ordinarily stratify only with the greatest difficulty, such as the unfertilized egg of *Nereis* and immature and unfertilized mature eggs of the starfish. The former cannot be fragmented since the chorion is too tough, but the latter readily separate into many pieces, spheres, or elongate cylinders (Fig. 6, *A*). The cylinders slowly round up again in sea water. *Cumingia* eggs may likewise be pulled apart into small fragments. *Arenicola* and *Phascolosoma* eggs,

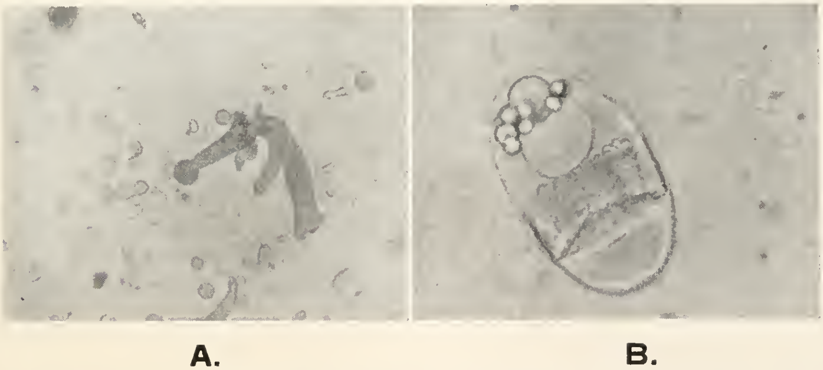

FIG. 6. (*A*) Unfertilized mature and immature starfish eggs (*Asterias forbesii*) fragmented by rotation for 10 minutes at 84,000 × g. (*B*) *Nereis limbata,* unfertilized immature eggs, rotated for 10 minutes at 84,000 × g.

unfertilized, stratify readily but do not pull into fragments because of the strength of the chorion, although there is some elongation.

Nereis eggs rotated 1300 R.P.S. (84,000 × g.) for 10 minutes are somewhat elongated (Fig. 6, *B*). They develop when fertilized, producing apparently normal swimming larvae. In sea water no redistribution of the granules occurs in the unfertilized eggs even after a period of twenty-four hours.

Arbacia eggs fertilized and centrifuged for 10 minutes at 84,000 × g., 29 minutes after fertilization (at 20° C.) become markedly stratified with even the red pigment completely thrown down. With ordinary forces (10,000 × g.), many of the red pigment granules stick to the egg surface. In some eggs the oil may be pulled through the fertilization membrane. Fertilized *Arbacia* eggs subjected to such high centrifugal forces at 5, 29, 42, and 55 minutes after fertilization are all markedly stratified and many of them develop into apparently normal-looking, free-swimming blastulae. A more careful study must be made to detect possible slight abnormalities in the larvae of " supercentrifuged " eggs, since it seems almost certain that eggs which are equipotential, nevertheless contain organ-forming substances that can be moved by these forces. With non-equipotential eggs the organ-forming substances should be readily moved.

The stroma from hemolyzed erythrocytes can be readily thrown down, as well as corpuscles " reversed " from hemolysis by addition of salt. Many uses in connection with erythrocyte behavior suggest themselves.

SUMMARY

The air turbine centrifuge, suitable for biological investigations with very high centrifugal forces, is described, together with some observations on living cells.

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THE GLOCHIDIAL CONGLUTINATES OF THE ARKANSAS FANSHELL, CYPROGENIA ABERTI (CONRAD)[1]

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The successful transfer of ripe glochidia from the maternal marsupium to the gills of the proper host fish constitutes one of the critical events of the life cycle of the fresh-water mussel. The difficulties of this transfer, which is accomplished largely as a matter of chance, are offset by the production of enormous numbers of glochidia, rather than by special structures or devices. It is, therefore, of interest to find a species of fresh-water mussel, *Cyprogenia aberti* (Conrad), the Arkansas fanshell, which has developed a special type of conglutinate that apparently serves to lure fish, since these conglutinates readily attracted fish, and when eaten by fish produced glochidial infections on the gills of these fish.

On February 2, 1932, a bright red, worm-like object about 1 mm. in length was observed projecting approximately 1.5 cm. from the exhalent syphon of the specimen of the Arkansas fanshell, in one of the experimental tanks. This mussel and others, both of the same and different species, which had been collected from the St. Francis River, Arkansas, during the preceding September and shipped to the laboratories at the University of Missouri, were living undisturbed in sand at the bottom of a large hatchery tank through which fresh water was constantly flowing. The anterior end of this red object was expanded into what appeared to be a definite cephalic portion and the whole structure occasionally waved back and forth. When removed from the mussel this object was found to be over 3 cm. in length and to be composed of glochidia in various stages of development, that is, the whole was a single glochidial conglutinate. The expanded "head-like" portion (Figs. 3 and 4) was pierced by one large opening, which in some specimens subsequently observed was more or less subdivided by masses of glochidia. On examination under the microscope it was found that the entire outer surface of the conglutinate was covered with ripe, fully-formed glochidia and that the central axial portion was made up of embryos and incompletely developed glochidia. The red color of the entire conglutinate was due to these central embryos, which contained more or less bright red material, the amount of the red substance varying inversely with the degree of development of the embryo. The mature glochidia were colorless, very transparent, and gave the outside of the conglutinate a frosted appearance. At the anterior end of this worm-like conglutinate, that is, the end first extruded from the mussel, the mature glochidia were found only on the outside, but they increased in numbers toward the posterior end of the conglutinate, which was composed almost entirely of mature ripe glochidia. The terminal portion (Fig. 5) of the posterior end of this conglutinate, therefore, had a white translucent appearance owing to the absence of the red immature embryos. The color of the entire conglutinate was, however, predominantly red, shading through light red to pink near the posterior end. On comparison with Ridgways' Color Standards (1912), the color of the conglutinate was noted as varying from garnet brown at the anterior end to nopal red near the posterior end. The measurements of five expelled conglutinates are given below (Table I).

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[1] The writer wishes to express his indebtedness to Dr. M. M. Ellis for his help in various parts of this investigation.

TABLE I

Dimensions of Expelled Conglutinates in Millimeters

Length of entire conglutinates	Minimum diameter	Maximum diameter exclusive of "head"	Width of "head"	Length of "head"
33.34	0.49	1.17	1.28	2.21
23.64	0.54	1.00	1.46	1.90
21.60	0.39	1.11	0.97	1.96
51.82	0.59	1.11	1.79	3.04
26.84	0.78	1.20	2.10	3.12
Average: 31.45	0.56	1.12	1.52	2.45

After discovering the first extruded conglutinate, 17 additional female specimens of *Cyprogenia aberti* were brought under observation and each of these individuals subsequently extruded red worm-like conglutinates. The ejection of a single conglutinate required from 3 to 12 hours and as far as was observed was not correlated with any particular external stimulus. When completely extruded the conglutinate fell to the sand alongside the mussel where it remained unless moved about by water currents. Within three or four days after the extrusion from the body of the mussel, undisturbed conglutinates at the bottom of the tank lost their color and the mature glochidia when examined were found to be weak or dying, as shown by their closing reactions when tested with physiological salt solution.

Sterki (1898) in a discussion of the anatomy of the closely related species of fresh-water mussel, *Cyprogenia irrorata* (Lea), noted the worm-like appearance of the conglutinates lying in place in the marsupial portion of the gill and described these conglutinates as worm-like cylinders which could be extracted *in toto* from the enclosing membrane. On dissecting gravid specimens of *Cyprogenia aberti* (Conrad), the writer noted the curved position of the marsupium which is characteristic of species of this genus and the arrangement of the conglutinate as figured by Ortmann (1912). When the marsupium was in place, the anterior or head-like portions of the ripe conglutinates were found to be formed by masses of glochidia lying in the top of each marsupial compartment at the point where this compartment joins supra-branchial passage. The flat posterior portion of the conglutinate filled the outer or distal section of the marsupium (see Figs. 6 and 7). During the extrusion of the conglutinate the entire structure was forced first into the supra-branchial passage and from there on out through the excurrent siphon.

The question arises whether the extrusion of these conglutinates as described in the specimens of the Arkansas fanshell is a normal procedure or whether it was merely a case of abortion. Lefevre and Curtis (1912, p. 137) state that " in *Obliquaria reflexa,* however, the conglutination persists, and the fully developed glochidia, still tenaciously adhering, are discharged from the marsupium in the cylindrical masses already described." These authors figured the expelled conglutinates of this species as curved cylindrical masses which they state " do not disintegrate even after lying in the water for some time." As *Obliquaria reflexa* and *Cyprogenia aberti* are rather closely related species, it would seem in view of the findings of Lefevre and Curtis that the extrusion of conglutinates by *Cyprogenia aberti* was not an abortion, particularly since these worm-like conglutinates of *Cyprogenia aberti* were covered with mature ripe glochidia which gave vigorous closing reactions when tested separately.

To eliminate the possibility of abortion or abnormal discharge of the conglutinates a second shipment of *Cyprogenia aberti* from Arkansas was made early in September, 1932, care being taken to insure the minimum disturbance of the mussels while in transit. As soon as these specimens arrived they were placed in a specially prepared hatchery trough through which fresh water flowed continuously and on the bottom of which was a layer of sand some 8 inches deep. The fanshells soon established themselves in the sand and as far as could be determined remained in good condition. Individuals of several other species of mussels were carried in this trough as controls and all mussels in the trough lived throughout the winter.

On February 15, 1933, the first red conglutinate was extruded from one of the specimens of the *Cyprogenia aberti,* and the spawning of this species continued during the next four weeks. Each female during this period extruded from 6 to 20 conglutinates. As these animals had not been disturbed since their arrival in September, that is, some five months previous, and as there had been no deaths in the group, nor among the control series of other mussels, it seems that this extrusion of conglutinates by the *Cyprogenia aberti* is a normal procedure and not an abortion due to some abnormal condition, particularly since the spawning period began in February both years.

As continuous thermographic records of water temperature were taken throughout the entire period these animals were under observation, and as the dissolved oxygen, carbon dioxide, carbonates, and pH were determined at regular intervals, correlations with the physical and chemical conditions of the water and the onset of the spawning period were looked for. Of the factors determined, temperature was the only one showing a significant change near the onset of the spawning activities.

The temperature charts showed that the average water temperature in the hatchery tank fell slowly through September to January, from 20° C. to 16° C. and that during February the average temperature rose rather rapidly to about 19° C. In view of the known relationship between temperature and spawning time of various other aquatic animals, and this rise in water temperature which occurred both years in February, it seems that the spring rise in water temperature is at least a contributing factor in initiating the spawning activities of the Arkansas fanshell.

Having established the fact that the formation and extrusion of these queerly shaped conglutinates are normal activities in this species of fanshell, and in view of the striking resemblance of these conglutinates as

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EXPLANATION OF PLATE I

FIG. 1. A single expelled conglutinate of *Cyprogenia aberti* (Conrad), showing ripe glochidia on the outside. Some free glochidia may be seen near the conglutinate.

FIG. 2. Five expelled conglutinates. Note the frosted appearance of the posterior portion of each due to the covering of ripe glochidia. Numerous free glochidia may be seen.

FIG. 3. Side view of anterior portion of conglutinate.

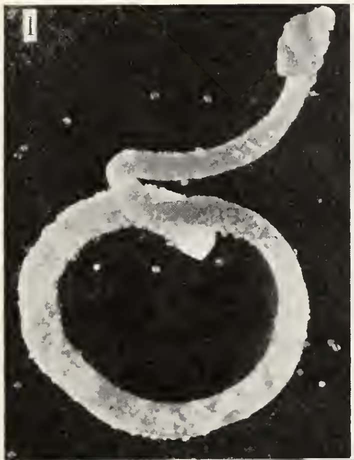
FIG. 4. Top view of anterior portion of conglutinate.

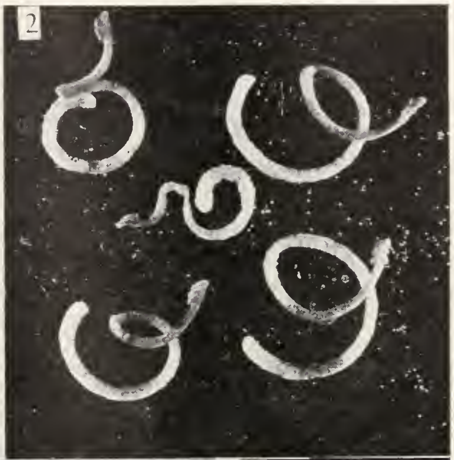
FIG. 5. Terminal part of posterior portion of conglutinate.

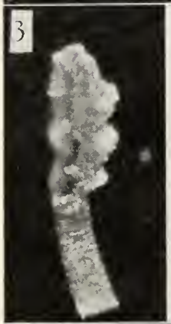
FIG. 6. Entire mussel lying in right valve. The left valve and left mantle have been removed. The arrangement of the conglutinates in the outer or marsupial gill can be seen.

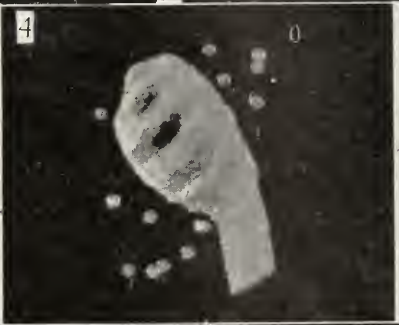
FIG. 7. Same mussel as Fig. 6. The left marsupium has been raised, exposing the inner gill, and below the lower half of the right marsupium.

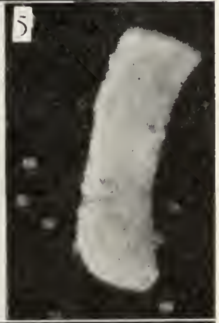
PLATE I

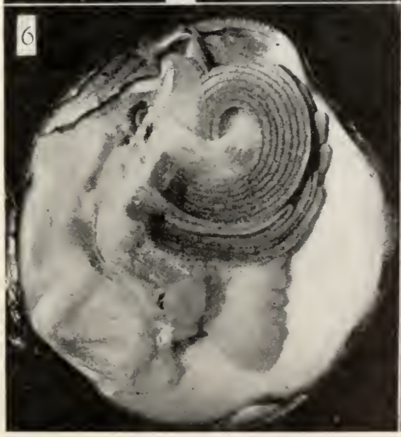


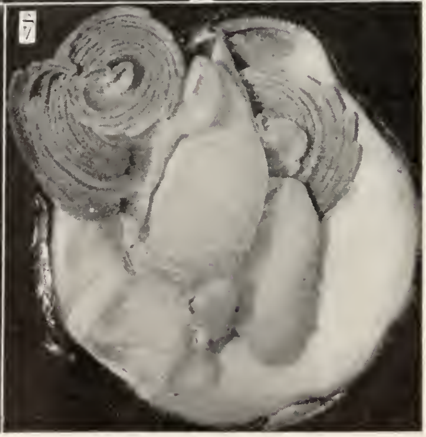












they waved back and forth from the exhalent syphons to tubificids or other bottom-inhabiting worms, some experimental tests were made to determine the reactions of fish to these brightly colored conglutinates, both during extrusion and after separation from the gravid mussel. Single individuals of gravid *Cyprogenia aberti* were placed in large glass battery jars, through which fresh water flowed continuously and which contained suitable layers of sand at the bottom. After the mussels had burrowed into the sand and were properly established, the extrusion and discharge of the red conglutinates proceeded as before, a medium-size goldfish (circa 3 inches long) was then placed in each jar and the reactions of the fish carefully noted. In every case the fish greedily ate any exposed conglutinates. The fish quickly seized incompletely extruded conglutinates and pulled them out of the exhalent siphon of the gravid mussel, and also picked up any discharged conglutinates lying on the bottom of the jar. Discharged conglutinates were readily eaten by other goldfish when placed on the bottom of the tank in which these fish were kept. As all of the fish used in these experiments were well fed, the avidity with which these fish selected and ate the conglutinates must be charged to a normal and not to a starvation reaction.

The goldfish which had fed on conglutinates were killed at intervals of from 15 minutes to 5 hours after the ingestion of the conglutinates. In every case glochidial infections on the gills were noted. Apparently the ripe glochidia, which are easily loosened from the outside of the conglutinate, attach themselves to the gills of the fish while the conglutinate is in the process of being swallowed. From these experimental findings the value of these brightly colored conglutinates to the species in the transfer of glochidia from gravid female to a host fish is evident.

The axial masses of immature glochidia and embryos passed on into the stomach, where they were rapidly disintegrated by the digestive juices in the course of an hour and a half. Fragments of glochidia were found in the duodenum five hours after the conglutinates were taken into the mouth.

These observations on the conglutinates of *Cyprogenia aberti,* and their usefulness as a device for the transfer of glochidia to the host fish, strengthen the suggestions of Coker, Shira, Clark, and Howard (1921, p. 85) concerning the function of the mantle flaps in the pocketbook, *Lampsilis ventricosa*. These writers state that " the conspicuous flaps which sometimes suggest the appearance of small fish, may serve as a lure to fish, bringing them into desirable proximity to spawners when glochidia are ready for extrusion." In this connection it may be added that in several species of fresh-water mussels belonging to the *Lampsilinæ* the marsupium and mantle margins in gravid females are more or less brightly colored with black, brown, or yellow, the gravid marsupia being partly extruded at times by these mussels. These colored structures may also serve as fish lures.

SUMMARY

1. The brightly colored worm-like conglutinates of *Cyprogenia aberti* (Conrad), the Arkansas fanshell, are described.
2. Experimental evidence is offered showing that these conglutinates serve as fish lures and thereby aid in the transfer of the ripe glochidia from the gravid female mussel to a host fish.

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THE COLORED CORPUSCLES OF THE BLOOD OF THE PURPLE SEA SPIDER, ANOPLODACTYLUS LENTUS WILSON

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In Europe, the cells of the circulating fluids of invertebrates have been given considerable attention. The data on the corresponding animals of America are much less complete. Of great interest, especially from an evolutionary viewpoint, are the cells of more or less fixed form in which the respiratory pigments, hemerythrin or hemoglobin, are enclosed. The other recognized respiratory proteins, chlorocruorin and hemocyanin, are reported as occurring only in solution in the blood or body fluids.

A survey of the literature yields little information on the blood cells of the pycnogonids. Cells of definite form, designated as "ballons," were described by Dohrn (1881) as occurring in the pycnogonids of the Gulf of Naples. These observations were supplemented by Cuénot (1891), who studied the pycnogonids in the vicinity of Banyuls. According to the latter author these cells contain an unidentified albuminoid which is either colorless or of a light neutral tint and does not change color on exposure to the air. The present report deals with the colored corpuscles of the sea spider, *Anoplodactylus lentus* Wilson (*Phoxichilidium maxillare* Stimpson), which is found in considerable numbers in the vicinity of Woods Hole.

The corpuscles of this animal range in color from light pink, deep pink, and lavender to purple and dark blue, and appear to be extremely specialized cells. The color of the blood, however, is blue.

VITAL OBSERVATIONS ON ENTIRE ANIMALS AND AMPUTATED LEGS

The blood spaces of the body of pycnogonids are divided into dorsal and ventral halves by septal membranes which are not limited to the body but run to the tips of the legs. The course of the circulation is outward in the inferior or ventral sinus and inward towards the heart in the superior or dorsal sinus. In forms like the species studied, with small bodies and long, slender legs, the circulation within the appendages is dependent largely on limb movements and on the contractions of the intestinal cæca which extend into the appendages.

Anoplodactylus is sufficiently small and translucent that the entire animal may be compressed under a large coverslip and the heart-beat and circulation observed under relatively high magnification. More satisfactory observations, however, may be made on amputated legs mounted in sea water. The cut end is quickly plugged by a clot and practically no blood is lost. A high dry lens or even a 2 mm. oil immersion can be used on many of these preparations. The contractions of the intestinal cæca persist for a long time and the contents of the sinuses surge back and forth with each pulsation, permitting observations on the corpuscles as they change position and orientation.

The corpuscles are extremely thin discs, irregularly oval or lenticular in outline, tapering at the ends into slender processes of varying length (Figs. 1 to 6), and in optical section these cells are seen to possess thickened, rounded margins. The nucleus is occasionally centrally located (Fig. 2), but usually is found in the thickened margin about midway between the two poles (Fig. 7). In the thin central region the pigment is scanty, becoming denser toward the periphery of the cell. The terminal processes do not contain pigment and may be readily overlooked in fresh preparations. The cells vary widely in size. These variations may be due to the degree of maturity of the corpuscles since the small cells usually possess less color.

In the smaller elements the pigment is uniformly distributed throughout the body of the cell; in the larger, it is marked off into irregular areas by clear bands which tend to run longitudinally, although transverse bands are not uncommon. The nature of these divisions is not clear.

As already noted, there is a wide range in the color of the corpuscles when observed either in the intact animals or in amputated appendages. Some of the variations may be due to differences in concentration of the pigment but there is some evidence that the deep blue represents the completely oxidized form, while the lavender and pink shades are caused by varying degrees of reduction of the pigment.

The cells are extremely flexible and may be folded longitudinally, twisted spirally, or folded transversely as they are subjected to pressure from the pulsating intestinal cæca. Recovery from such deformation is rapid. The tips of the terminal processes are apparently adhesive in nature, and cells frequently become attached to the walls of the sinuses by one or both ends and sway and twist about as the blood surges back and forth.

SUPRAVITAL OBSERVATIONS

Sufficient blood for supravital observations on the corpuscles may be obtained by gently pressing the amputated legs. The blood is frequently mixed with ova and portions of the cæcal epithelium, but excellent preparations of almost pure blood may sometimes be obtained. The supravital preparations permit a more critical study of the morphology of the corpuscles, but degenerative changes occur more rapidly than when the blood is in the sinuses of the leg.

No formed bodies other than the nucleus can be distinguished in the fresh cells. In stale preparations the pigment loses its homogeneity and denser globules of varying size suspended in a paler fluid appear (Fig. 8). If the globules are large, no Brownian movement is discernible but smaller bodies may exhibit intense activity. The clear bands appear to form definite boundaries within the cell and the vibrating globules are unable to pass across them.

In sealed coverslip preparations which have stood for several hours many cells develop fine beaded processes on their surfaces. These processes contain pigment and usually show Brownian movement. Minute globules may separate from their free ends and persist for some time as free bodies in the plasma.

In hypertonic solutions the cells are shortened and thickened and irregular in outline (Fig. 10), resembling in some respects the crenated corpuscles of the vertebrates. In hypotonic solutions, produced by adding distilled water at the margin of the coverslip, the cells swell, lose their thin, flattened form (Figs. 11 and 12) and eventually become

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EXPLANATION OF PLATE

All drawings are from fresh preparations and were outlined at the same magnification (× 1500) by means of a camera lucida. The nuclei appear as light areas.

Plate I

Explanation of Figures

1 to 6. Surface views of colored corpuscles of *Anoplodactylus lentus,* showing variations in size, form, and distribution of the pigment.

7. Optical section of a corpuscle viewed on edge drawn at the level of the marginal nucleus.

8. A corpuscle showing the formation of dark globular masses which frequently appear in sealed preparations which have stood for several hours.

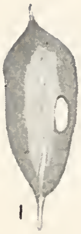
9. A corpuscle after supravital staining with neutral red and Janus green B. No characteristic neutral red bodies or mitochondria could be demonstrated. Dark, refractile granules of varying size, exhibiting active Brownian movements, appeared after the application of the dyes.

10. A corpuscle in a hypertonic solution viewed on edge showing a condition comparable to crenation in the vertebrate erythrocyte.

11 and 12. Early stages in the transformation of the thin flattened corpuscles into spheres.

13, 14, 15, and 16. Spherical corpuscles, usually containing induced granules of varying size, were produced by several methods. The occurrence of the pigmented content in separate masses is probably correlated with the presence of the clear septa present in many normal corpuscles.

17. Corpuscle after exposure to dilute hydrochloric acid, showing loss of pigment and the appearance of dense refractile granules.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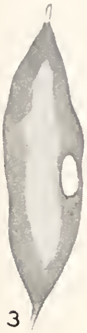
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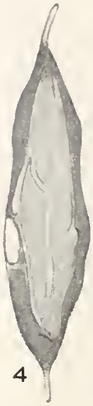
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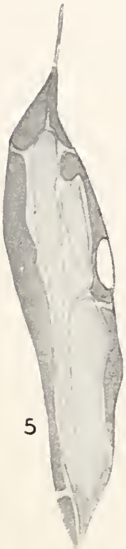
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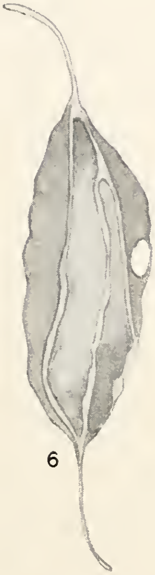
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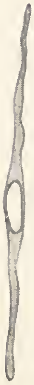
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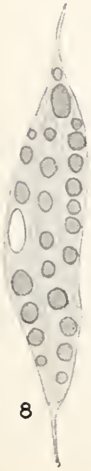
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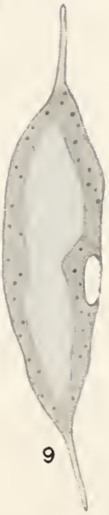
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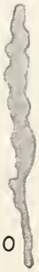
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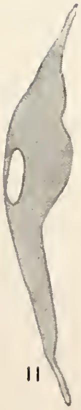
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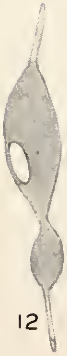
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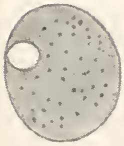
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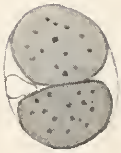
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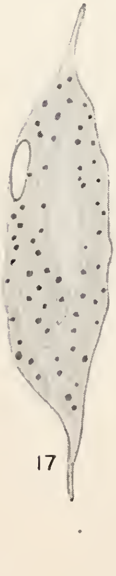
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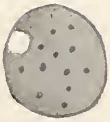
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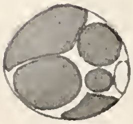
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converted into spheres (Figs. 13, 14, and 15). Such cells usually contained induced bodies of varying size which are in active Brownian motion. In these spherical cells (Figs. 15 and 16) the pigment is frequently divided into separate masses, a condition which is probably correlated with the presence of clear bands in the normal corpuscles.

Fresh preparations stained supravitally with neutral red, Janus green B, or brilliant cresyl blue reveal no additional cytoplasmic constituents such as mitochondria, neutral red bodies, or patterns of reticulation. In all cases, however, the large, more deeply pigmented cells contained densely colored bodies of varying size (Fig. 9), which exhibited different degrees of Brownian activity. This reaction to vital dyes appears comparable to that obtained in stale preparations, although the induced bodies are never so large. It is probably the result of injury. The characteristic color of any of the dyes was never obtained; it was masked by the natural pigment of the cell.

The Nature of the Intracellular Pigment

The color range of the corpuscles in entire animals and in amputated legs appeared the same. Attempts were made to determine the nature of the pigment and the factors producing the variations in color but the results were not at all conclusive. Only minute amounts of blood could be obtained by expressing the fluid from the legs so that all tests were carried out on the slide by drawing the various solutions under the coverslip.

It was obvious that the concentration of pigment varied in individual cells, probably directly with the degree of their maturity. The hydrogen ion concentration of the medium did not appear to influence the color of the pigment. Solutions of hydrochloric acid in sea water (pH 3.0) did not cause any change in color, but greater acidity caused a loss of pigment from the cell, probably due to cytolysis. Following the extraction of the pigment, numerous dense, refractile bodies were noted in the portions of the cell from which pigment was lost (Fig. 17). Solutions of sodium hydroxide (pH 9.0) also produced no color change but the cells rapidly assumed the spherical form (Fig. 17).

Reducing agents produced more positive results. Sodium hydrosulphite was applied in pulverized form at the margin of the coverslip and sufficient sea water added so that the solution might be drawn under the slip. Changes in color were noted after 15 minutes. Cells which were purple and deep blue changed to light brown and brownish yellow. Lavender cells became pink and pink cells colorless. After two hours the majority of the cells were colorless but a number of light yellow and pale pink cells could still be seen in the field. After four hours the reduction of the color was almost complete. The reduction of the pigment was also accomplished by applying potassium cyanide in a similar manner. The more deeply colored cells in this instance faded to various shades of slate and gray before becoming colorless. Decolorization was frequently complete in about three quarters of an hour. Attempts to reoxidize by air the pigment in preparations treated with sodium hydrosulphite were unsuccessful, and questionable results were obtained with potassium ferricyanide. This may have been due to limitations of the method employed in handling such small amounts of blood.

Fresh preparations sealed with vaseline and kept for as long as forty-eight hours were not appreciably changed in color by the exclusion of air. Blood drawn from animals which were sealed in vials of boiled sea water until all activity had ceased and they had become limp seemed to contain a lower percentage of purple and blue cells than normal animals. The blood picture as regards color of the corpuscles, however, is very variable and comparisons are difficult. Macroscopically the blood seemed lighter in color, and when exposed to air seemed to grow darker.

Microspectroscopic examination of individual cells from dry unstained smears did not demonstrate any definite absorption bands for the pigment preserved in this manner. These preliminary tests are obviously inadequate to determine the identity or nature of the pigment. There is, however, some slight evidence that it may undergo reversible oxidation-reduction changes or play some rôle in oxygen transfer.

The variations in color of the corpuscles in the circulation appear to depend on two factors: the relative concentration of the pigment within the cells and the degree of its reduction or oxidation. A third possibility—the presence of another pigment, especially in the dark blue cells which do not decolorize in the same manner as the lighter cells—should not be overlooked.

One of the most widely distributed blue pigments in the animal kingdom is hemocyanin. This substance, however, has not been reported within cells of the circulating fluids but has always been found dissolved in the plasma. Another, as yet unidentified, blue-purple pigment has been reported as occurring in the blood and tissues of *Chromodoris zebra* and related species (Crozier, 1914, 1922) and some of the physical and chemical properties of this pigment have been described by Preisler (1930). Further studies on the intracorpuscular pigment of the blood of *Anoplodactylus* are needed in order that its chemical nature and functional significance may be ascertained.

I am indebted to Professor A. C. Redfield for helpful suggestions during this study.

SUMMARY

The blood of the sea spider, *Anoplodactylus lentus,* contains numerous pigmented corpuscles. The cells are thin, flexible discs of lenticular or irregularly oval outline with a marginally located nucleus. The poles of the cells are elongated into slender processes which are adhesive.

The corpuscles range in color from pink, lavender, and purple to deep blue. The pigment may be homogeneously distributed or limited to areas marked off by clear bands.

These cells are readily deformed by changes in the concentration of the medium. Staining reactions characteristic of vertebrate erythrocytes are not produced by exposure supravitally to neutral red, Janus green B or brilliant cresyl blue.

Preliminary tests indicate that variations in the color of the pigment are not dependent on hydrogen ion concentration.

The pigment may be decolorized with potassium cyanide or sodium hydrosulphite, but attempts to restore the color were inconclusive. The small amounts of blood obtainable render tests of this nature difficult.

This pigment, obviously neither hemoglobin or hemerythrin, occurring intracellularly in the blood of an invertebrate, and possibly of respiratory significance, merits the attention of physiologists.

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STUDIES ON THE CILIATES FROM FRESH WATER MUSSELS

I. The Structure and Neuromotor System of Conchophthirius anodontæ Stein, C. curtus Engl., and C. magna sp. nov.

GEORGE W. KIDDER

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Although the ciliate commensals of the various fresh water mussels exist in great abundance and offer excellent material for a cytological study, very little attention has been paid to them. The greatest number of these organisms belong to the genus *Conchophthirius*. Indeed, the type species *C. anodontæ* (Ehr.) Stein is found in one of these mussels. Since the work of Schuberg in 1889 there has been but one paper of merit,—that of Raabe (1933),—dealing extensively with this genus, and no work, as far as I am aware, of a strictly cytological nature. Raabe has given an excellent review of the literature and an account of certain morphological features of four species of *Conchophthirius*. Employing the silver nitrate method of Klein (1926*a, b*), he was able to establish the existence of a previously undescribed species, *C. unionis*, and to place the ciliate called by Claparède and Lachmann (1858) *Plagiotoma acuminata* in the genus *Conchophthirius*. He has based his specific comparisons chiefly on the pattern of the " silverline system " and only noted those extra characteristics which were easily observed in life. Schuberg (1889), while giving an excellent description of *Conchophthirius anodontæ* and *C. steenstrupii,* did not recognize Engelmann's (1862) *C. curtus,* but it is quite obvious, if one is to observe even cursorily the forms from various species of *Anodonta*, that in this judgment Schuberg was wrong.

To summarize briefly the existing species of the genus *Conchophthirius* so far known to the literature, I shall list only those which I consider valid. For a more extensive discussion the reader is referred to the work of Raabe (1933). The list is as follows: *Conchophthirius anodontæ* (Ehr.) Stein 1861, *C. steenstrupii* Stein 1861, *C.* (*Plagiotoma*) *acuminata* Claparède and Lachmann 1858, *C. curtus* Engelmann 1862, *C. antedonis* André 1910, *C. discophorus* Mermod 1914, *C. mytili* deMorgan 1925, *C. unionis* Raabe 1933, and *C. caryoclada* Kidder 1933*a*.

Attention should be directed to the ciliate called *Conchophthirius metchnikoffi* by Certes (1891), which was later removed to the genus *Phascodinium,* order Heterotrichida, and to *Conchophthirius elongatus* and *C. lamellidens* of Ghosh (1918). The last two species cannot be considered seriously, as the descriptions and illustrations of Ghosh are far too brief and sketchy to allow one to judge their validity.

It is the purpose of this paper to present the results of a cytological investigation of the structure and neuromotor apparatus of members of the genus *Conchophthirius* encountered in the examination of seven species of mussels taken from fresh water lakes in Massachusetts and New York. It is my hope that this work will lead to a clearer understanding of the relationships which exist between the external fibrillar system, as described so carefully by Raabe (1933), and the internal fibrillar system demonstrated by the methods here employed.

The species with which I shall deal are *Conchophthirius anodontæ* (Ehr.) Stein, *C. curtus* Engl. and a hitherto undescribed species, which, because of its large size, I shall call *Conchophthirius magna.*

The basic structural differences between these species will be pointed out in the general descriptions. Because of the very close agreement of their neuromotor systems, these elements in all three species will be considered together, attention being directed only to points of difference.

This investigation was carried on at the Marine Biological Laboratory at Woods Hole, Massachusetts. I wish to express my indebtedness to W. J. Clench, Curator of Molluscs in the Museum of Comparative Zoology of Harvard University, who very kindly identified the species of mussels used in this work.

Material and Methods

The three species of ciliates to be described are found in the fluids of the mantle cavities and creeping about over the gills and palps of various species of fresh water mussels. The infections vary from only a few ciliates per mollusc in the cases of *Anodonta implicata, A. cataracta, Lampsilis radiata, L. cariosa* and *Alasmidonta undulata* to hundreds in the cases of *Anodonta marginata* and *Elliptio complanatus.*

In an attempt to determine the exact location of the ciliates in their hosts many mussels were opened carefully and parts of the mantle, gills, and palps dissected out and examined. In the cases of *C. curtus* and *C. magna* no definite location seemed to be preferred as ciliates were found on all of the exposed surfaces and also swimming freely in the mantle fluids. But in the case of *C. anodontæ* there invariably occurred a localization of organisms on the non-ciliated surface of the palps. The ciliates found here exhibited entirely different reactions in regard to movement from those found elsewhere. When the palp was removed from the mollusc and carefully flattened out in a Syracuse dish, the non-ciliated surface appeared mottled with *C. anodontæ*. In contrast to the ceaseless activity normally encountered, they remained extremely quiet, attached by the cilia of their ventral surfaces. Examination under high magnifications showed the cilia of the peristomal region alone in vigorous motion while the body cilia waved listlessly. When dislodged from the palp the organisms moved rapidly and continued this activity until they became moribund.

There appears to be a fair degree of host specificity in this group *Conchophthirius magna* was found only in *Elliptio complanatus* and was always accompanied by *C. anodontæ*, while *C. curtus* was found in *Anodonta marginata*, *A. implicata*, *A. cataracta*, *Lampsilis radiata*, *L. cariosa* and *Alasmidonta undulata*. Although all of these mussels may be found side by side buried in the muddy bottom of the lake, it is only occasionally that there is cross infection in nature. I have found small numbers of *C. curtus* associated with *C. anodontæ* in *Elliptio complanatus* and likewise a few *C. anodontæ* with *C. curtus* in *Anodonta marginata*. I have never found *C. magna* in any host but *Elliptio complanatus*. The reason for the segregation in nature is not clear, because no such condition is found in the close association of the hosts in the laboratory tanks. When various species of mussels are placed in a small aquarium cross infection results in a few days. I have examined the contents of *Anodonta* and *Elliptio* which have been kept together for a week and have found about equal numbers of *Conchophthirius anodontæ* and *C. curtus* in every specimen. There seems to be no doubt that cross infection is brought about by the ciliates leaving one host and swimming to the next, as pipette samples from the bottom of the aquarium rarely fail to reveal the presence of one or more organisms that are free from a host.

When washed free of the mantle cavity the ciliates swim about actively, but I have been able to keep them alive in a Syracuse dish for from twelve to twenty-four hours only. The movements gradually cease and death ensues although plasmolysis seldom occurs for a considerable time. Dishes containing the dead ciliates have been kept for many days with very little noticeable change in the body form.

The technical methods here employed were similar to those used in the study of *Conchophthirius mytili* (Kidder, 1933*b*), but because of the abundance of material it was found possible to fix and stain hundreds of organisms on each cover glass. The ciliates were washed into a Syracuse dish and an albuminized cover glass immersed in the fluid. After a preliminary agitation with a pipette the ciliates were allowed to settle on the cover glass which was then removed with clean forceps.

The excess fluid was drained off and the cover glass quickly immersed in the desired fixing fluid. This method proved very satisfactory as the adherent powers of these ciliates caused them to remain in close contact with the albuminized surface.

In addition to the method of Klein (1926*a, b*) for a study of the external fibrillar system, the most useful method was found to be a 4-hour fixation in strong Flemming's fluid, a thorough washing in running water and staining the long method with Heidenhain's hæmatoxylin. Differentiation was carried out in a 10 per cent solution of hydrogen peroxide. After this technique the fibers of the external and internal systems remain black while the cytoplasm is a clear yellowish gray and the nuclei and food bodies are a bluish gray. This technique has proved invaluable both in this study and in the past (Kidder, 1933*b*).

The wet silver method of Gelei and Horváth (1931) gave fairly good results in demonstrating the internal fibers of the peristomal basket and the pharynx, but I have not been able to obtain quite as sharp a differentiation of external fibers with this technique as with that of Klein or Heidenhain's hæmatoxylin destained with H_2O_2.

For a study of the endoplasmic granules the best results were obtained by the use of the Borrel stain following Schaudinn's fluid.

For nuclear structures Heidenhain's hæmatoxylin and the Feulgen nucleic acid reaction following Schaudinn's fluid gave very good results. The former stain was also used after Bouin's, Flemming's and Champy's fluids.

Neutral red and Janus green B were used vitally but proved to be of very little value in this study.

Description of Species

Conchophthirius anodontæ Stein 1861

(Fig. 1)

The body outline as seen from the dorsal or ventral aspect is ovoid, being slightly more pointed at the anterior end. A lateral view shows this ciliate to be concave ventrally and slightly convex dorsally. The dorso-ventral axis is somewhat over one-third that of the antero-posterior axis. The left margin of the body forms an arc but the right side is slightly concave in the region of the buccal cavity.

The average size is $103\,\mu \times 69\,\mu$. The extremes of length were found to be from $125\,\mu$ to $65\,\mu$, while the extremes of width were from $86\,\mu$ to $47\,\mu$.

The cytostome is located on the dorsal surface in the anterior third of the body near the right margin. Anterior to the peristomal depression is an overhanging projection formed from the anterior body region.

The cytostome opens immediately under this projection and leads into a tubular pharynx which proceeds into the cytoplasm in a slightly anterior direction. The roof of the pharynx is provided with a single row of rather long cilia. The pharynx is lined with circular fibers (Fig. 1; Fig. 6*A*, *ph.*) and is contractile. It opens directly into an exceedingly long and recurved gullet (Fig. 1, *gul.*; Fig. 6, *g.*), which is a permanent path in the endoplasm and can easily be demonstrated in living and stained organisms. It is small in diameter but is quite distensible and can accommodate food bodies of considerable size. Schuberg (1889) described the intake of food but was unable to trace the gullet for any great distance. In all of my observations I have been able to demonstrate the ending of the gullet just posterior to the macronucleus (Fig. 1). The gullet sharply marks off from the rest of the body the loosely granular digestive endoplasm. I have never seen large food vacuoles outside of this region which is similar to though more extensive than the "food basket" of *Cryptochilidium bermudense* as described by Powers (1933*a*).

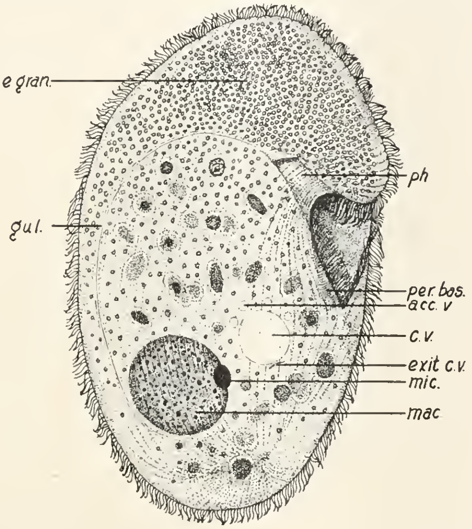

Fig. 1. *Conchophthirius anodontæ* Stein. Dorsal view. × 600.

acc. v., accessory vacuoles; *c. v.*, contractile vacuole; *e. gran.*, endoplasmic granules; *exit c. v.*, aperture through which the contractile vacuole empties; *gul.*, gullet; *mac.*, macronucleus; *mic.*, micronucleus; *per. bas.*, peristomal basket; *ph.*, pharynx.

The peristomal field is naked and extends posteriorly from the cytostome as a rather flat V-shaped area. It is lined with a net of fibers from which originate the ventral fibers of the peristomal basket. This basket (Fig. 1, *per. bas.*; Fig. 6, *p. b.*) is a depression between the dorsal lip, the edge which overhangs the peristome throughout its length, and the peristomal field. The lip is supplied with a row of cilia somewhat longer than those of the body. From the longitudinal fiber at the base of this row arise the dorsal fibers which line the peristomal basket.

The cilia of the body are arranged in longitudinal rows lying in grooves. The grooves are especially noticeable in the extreme anterior portion of the body where the cytoplasm is clear. The rows originate in a wide ventral suture (Fig. 6, *E, v. s.*) near the anterior end, as described by Raabe (1933), and terminate in a whorl near the posterior end on the dorsal surface (Fig. 6, *E, d. s.*). The dorsal suture of *Conchophthirius anodontæ* is a very small area as contrasted to the extensive sutures of the other species to be described later. The rows of cilia on the ventral surface are slightly more numerous than those on the dorsal surface, while the cilia themselves are much denser on both surfaces toward the anterior end than toward the posterior.

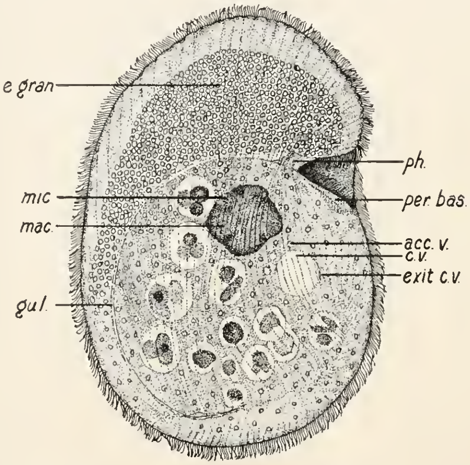

FIG. 2. *Conchophthirius curtus* Engl. Dorsal view. × 600.
acc. v., accessory vacuoles; *c. v.*, contractile vacuole; *e. gran.*, endoplasmic granules; *exit c. v.*, aperture through which the contractile vacuole empties; *gul.*, gullet; *mac.*, macronucleus; *mic.*, micronucleus; *per. bas.*, peristomal basket; *ph.*, pharynx.

The anterior region contains a zone of endoplasmic granules, which are quite large in this species (Fig. 1, *e. gran.*) and appear as highly refractile spheres in life. They react strongly with the indigo carmine of the Borrel mixture, appearing as a very blue shell with a less dense center. After the various silver methods this zone is quite blackened (Fig. 6, *E*). The granules are not strictly confined to this zone but are scattered sparsely throughout the rest of the endoplasm. They do not appear in the ectoplasmic layer.

The nuclei of *Conchophthirius anodontæ* are quite characteristic. The macronucleus (Fig. 1, *mac.*) is smoothly spherical and normally lies in the posterior third of the body always within the digestive endoplasm. It measures about 30 μ in diameter. It appears to be quite solid and in the resting condition is filled with spherical granules of varying size. I have never seen it distorted by adjacent food bodies to the extent observed in the two species to be described presently.

The micronucleus is very large and lies in a depression in the macronucleus (Fig. 1, *mic.*), seeming to be surrounded by the macronuclear membrane. I do not believe this to be the case, however, for at the onset of fission the micronucleus moves away from the depression, leaving it intact.

Just posterior and to the left of the peristome is to be found a single contractile vacuole (Fig. 1, *c. v.*). Varying numbers of accessory vacuoles (Fig. 1, *acc. v.*) form a ring about it and contribute to its volume as it fills during diastole. Dorsal to and in connection with the contractile vacuole is a well-defined slit-like structure (Fig. 1, *exit c. v.*) through which the contents of the vacuole empty to the outside on systole. This structure and its relationships will be discussed in detail later.

Conchophthirius anodontæ is found in great abundance in the mantle cavity, on the gills, and especially on the non-ciliated surface of the palps of *Elliptio complanatus* (Say) in 100 per cent of the molluscs examined. The hosts were taken from fresh water lakes in the vicinity of Woods Hole, Massachusetts. *C. anodontæ* is to be considered as a commensal since the food vacuoles are filled with algæ, bacteria, and sloughed-off epithelial cells of the host.

Conchophthirius curtus Engl. 1862

(Fig. 2)

In shape this species is similar to the preceding one except that the anterior and posterior ends are more bluntly truncate. The average width is proportionately greater than that of *C. anodontæ* and the average length proportionately less. The measurements of one hundred individuals gave the average length as 97 μ while the average width was 65 μ. The extremes of length were from 123 μ to 61 μ and the extremes of width were from 92 μ to 51 μ.

In general the peristomal region is like that of *C. anodontæ* except that the peristomal field does not extend as far posteriorly, and the pharynx (Fig. 2, *ph.*) is less conspicuous and pointed more directly toward the left margin. The gullet (Fig. 2, *gul.*) is similar but slightly more extensive than in the preceding species. The peristomal basket (Fig. 2, *per. bas.*) is smaller than in *C. anodontæ*.

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The body cilia are disposed in rows, slightly closer together than those in *C. anodontæ* (compare Fig. 6, *D* and *E*), which originate from a broad ventral suture at the anterior end of the body and terminate in a long dorsal suture on the posterior dorsal surface. This dorsal suture extends nearly the width of the body and is made up of an irregular net of fibers among which are situated large basal bodies. From these basal bodies arise long cilia. My observations on this region are entirely in agreement with those of Raabe (1933) on the same species. Because of the length of the dorsal suture the ciliary rows do not come together in a whorl, as in *C. anodontæ,* but are more evenly spaced along its length.

There is a distinct difference in the appearance of the zone of endoplasmic granules in *C. curtus* and *C. anodontæ*. The granules are similar in size and staining reaction but are much more closely packed in *C. curtus,* nor does the zone extend as far out toward the anterior end of the body (Fig. 2, *e. gran.*; Fig. 6, *G, e. z.*) as in *C. anodontæ*. This difference is striking and can be noted at once even in living organisms. Engelmann (1862) called attention to this and gave it as a specific characteristic of *C. curtus*. Only a few of the endoplasmic granules are found scattered about in the middle and posterior regions.

The macronucleus of *Conchophthirius curtus* is located normally just posterior to the gullet, in the middle of the body (Fig. 2, *mac.*). It is much smaller than that of *C. anodontæ,* measuring from 18 μ to 20 μ in diameter. In contrast to the solid condition of the macronucleus described above, this body appears to be quite amorphous. In life it can be seen to change its shape as food bodies are pressed against it. When external pressure is applied to the organism, causing it to break, the macronucleus goes to pieces very quickly, a condition only occurring after long exposure to the external fluids in the case of *C. anodontæ*.

The micronucleus (Fig. 2, *mic.*) is extremely minute and in the resting condition is embedded in the macronucleus. At this stage I have never been able to detect it in the living organism, but only in favorably stained preparations. It can be demonstrated quite regularly after the Feulgen nucleic acid reaction as it reacts a little more intensely than the chromatin of the macronucleus and appears to be surrounded by a halo. So small is this cell element that a thorough knowledge of its structure and position was gained only after a careful study of its activity during fission. Engelmann (1862) describes the macronucleus of *C. curtus* as possessing one or two "nucleoli." I have never found more than one micronucleus in this species and I doubt very much if what Engelmann saw were micronuclei or that even one of them was a micronucleus. In life the food bodies of varying size so obscure the nuclei that in my opinion no final conclusion as to the nature and number of a cell element so minute as this could be reached without recourse to careful staining techniques. Raabe (1933) figures a micronucleus in *C. curtus* almost as large as that of *C. anodontæ,* his drawing being made from *life*. Certainly the American variety of *C. curtus* will not lend itself to such simple observation.

The contractile vacuole (Fig. 2, *c. v.*) is similar in size and location to that of *C. anodontæ*. I have never found it in the posterior position described by Engelmann (1862). It is accompanied by a slitlike opening (Fig. 2, *exit c. v.*) similar to that found in *C. anodontæ.*

Conchophthirius curtus occurs in great abundance in the mantle cavity of *Anodonta marginata* Say in 100 per cent of the molluscs examined. It also occurs in fewer numbers in *Anodonta implicata* Say, *A. cataracta* Say, *Lampsilis radiata* (Say), *L. cariosa* (Say) and *Alasmidonta undulata* (Say). In nature it usually occurs alone in these mussels but may occasionally be accompanied by a very few *Conchophthirius anodontæ*. The hosts were taken from fresh water lakes in the vicinity of Woods Hole, Massachusetts, and from Lake Chautauqua, New York.

This species should also be considered as a commensal, since no indication of cell destruction of the host was noted, the food vacuoles containing algæ as well as sloughed-off epithelial cells.

Conchophthirius magna sp. nov.

(Fig. 3)

The body outline of this species is oval as viewed from the dorsal or ventral surfaces, the anterior end being somewhat pointed and wider than the evenly rounded posterior extremity. The peristomal area is seen on the right margin as an indented region extending posteriorly in the form of a narrow V. When viewed from a lateral aspect, this ciliate is seen to be very much flatter than either of the preceding species.

In size it is strikingly different from *Conchophthirius anodontæ* and *C. curtus*. The average length of two hundred individuals was found to be 180 μ while the average width was 95 μ. The range of length was from 203 μ to 123 μ and the range of width from 116 μ to 63 μ.

In life *C. magna* is easily identified both by size and because it is slightly clearer and more transparent than *C. anodontæ*. Also its movements, both swimming and creeping, are less jerky and more continuous than the latter species.

The pharynx (Fig. 3, *ph.*) is shorter than the pharynx of *C. anodontæ*, but the gullet (Fig. 3, *gul.*) is extremely long and recurved toward the right side. While the gullet roughly marks off the digestive area, the food vacuoles are not so strictly limited to this region as in the preceding species. I have never found any food bodies in the extreme anterior portion but occasionally a few are found to the left of the gullet among the endoplasmic granules. The peristomal basket (Fig. 3, *per. bas.*) is rather shallow and much longer than in *C. curtus.* The same type of structures as described for *C. anodontæ* are found in the peristomal field.

The body cilia are much finer and more closely set than the body cilia of either *Conchophthirius anodontæ* or *C. curtus.* The ciliary rows are exceedingly close together on both surfaces (Figs. 6, *B* and *C*). They originate in a rather broad ventral suture (Fig. 6, *C, v. s.*) toward the anterior end and terminate in a long dorsal suture (Fig. 6, *B, d. s.*) near the posterior end. This dorsal suture is of the same type as that of

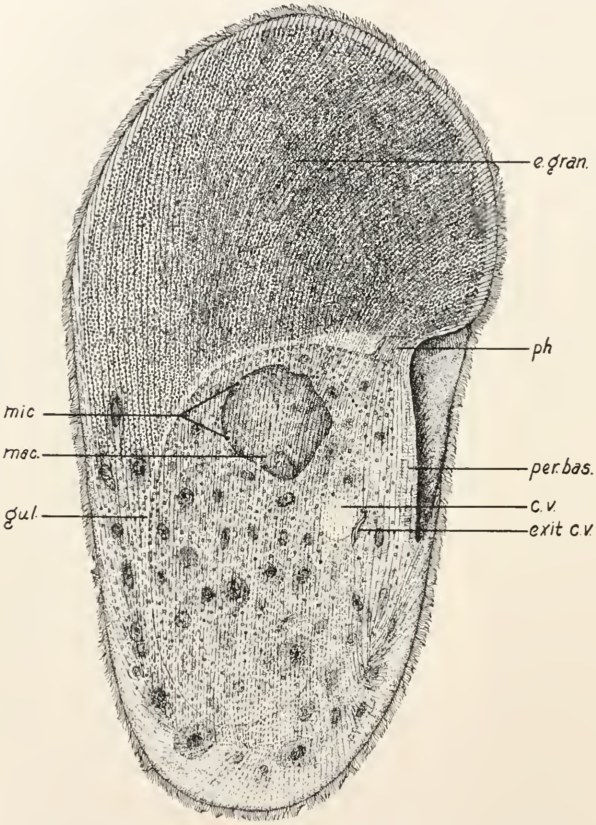

FIG. 3. *Conchophthirius magna* sp. nov. Dorsal view. × 600.
c. v., contractile vacuole; *e. gran.*, endoplasmic granules; *exit c. v.*, aperture through which the contractile vacuole empties; *gul.*, gullet; *mac.*, macronucleus; *mic.*, two micronuclei; *per. bas.*, peristomal basket; *ph.*, pharynx.

Conchophthirius curtus, although it is supplied with a greater number of large basal bodies from which long cilia originate. The region about the dorsal suture is naked except for these cilia. The longitudinal fibers of the ciliary rows proceed to the suture where their lack of basal bodies sharply defines a posterior naked zone (Fig. 6, *B, p. z.*). This area is much larger than in *C. curtus.*

The endoplasmic granules (Fig. 3, *c. gran.*) are about one-half the size of those in the preceding species. They are closely packed and fill the anterior third of the body, leaving a narrow, clear cytoplasmic layer. These granules are quite characteristic and by their relative size and extent it is possible to recognize the species immediately. A few of the endoplasmic granules are found scattered around the food vacuoles in the middle and posterior portions of the body.

The macronucleus (Fig. 3, *mac.*) is rather irregular in outline and is located just posterior to the gullet midway between the lateral edges. It measures about 25 μ to 30 μ in diameter. Like the macronucleus of *Conchophthirius curtus,* it is quite labile and is easily indented by the crowding food bodies. The chromatin is dispersed in very fine granules and numerous lighter areas give the macronucleus a slightly evacuolated appearance.

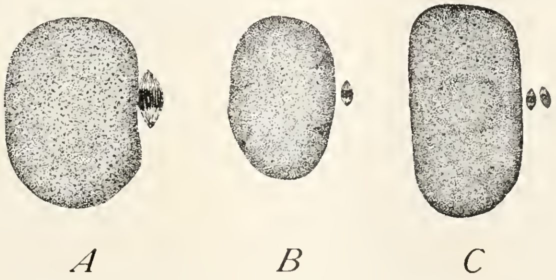

FIG. 4. The nuclear appearance during fission. × 900.

A. Conchophthirius anodontæ Stein. The extremely large micronucleus is in full metaphase.

B. Conchophthirius curtus Engl. The spindle is seen to be very much smaller than in *A*. The macronucleus still shows signs of its folded condition.

C. Conchophthirius magna sp. nov. The two spindles are seen to be about the same size as the spindle of *C. curtus.*

Embedded in the macronucleus are two minute micronuclei. These cannot be seen in the living state but can be demonstrated in the resting condition after a well-differentiated hæmatoxylin stain or after the Feulgen reaction. During fission their demonstration is most easily brought about as they then emerge from the macronucleus. Figure 4 illustrates the relative sizes and positions of the metaphase spindles of the three species here described.

The number of micronuclei in *Conchophthirius magna* appears to be not invariable. About 10 per cent of the well-prepared specimens were found to possess but one micronucleus, whereas in the remaining 90 per cent two were clearly discernible. This percentage held for the dividing organisms studied, as I shall show in a later paper.

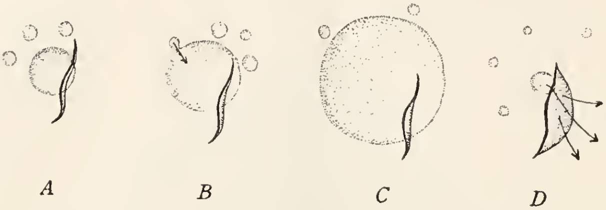

FIG. 5. Diagram representing the action of the contractile vacuole, the accessory vacuoles, and the aperture during diastole and systole.

A. Contractile vacuole slowly filling. The accessory vacuoles are large and the aperture is closed.

B. and *C.* Further enlargement of the contractile vacuole and fusion with the accessory vacuoles.

D. Systole of the contractile vacuole. The aperture opens and the fluid is poured to the outside. Small accessory vacuoles are again making their appearance.

The contractile vacuole (Fig. 3, *c. v.*) of *Conchophthirius magna* is similar in position though smaller in size than those of *C. anodontæ* and *C. curtus.* Like the species already described, *C. magna* possesses a slit

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FIG. 6. Photomicrographs.

A. Conchophthirius anodontæ Stein. Ventral view of an organism fixed in strong Flemming's stained with Heidenhain's hæmatoxylin and differentiated with H_2O_2. The photograph was taken at a mid-region focus. Note the fibers of the pharynx and peristomal basket. Also the cilia within the pharynx. × 630.

B. Conchophthirius magna sp. nov. Dorsal view of an organism prepared by Klein's method. The anterior region of the endoplasmic granules always become very heavily impregnated with the colloidal silver. The organism is somewhat flattened. × 420.

C. Conchophthirius magna sp. nov. Ventral view of an organism prepared as in *B.* to illustrate the distribution and number of ciliary rows and the ventral suture. × 420.

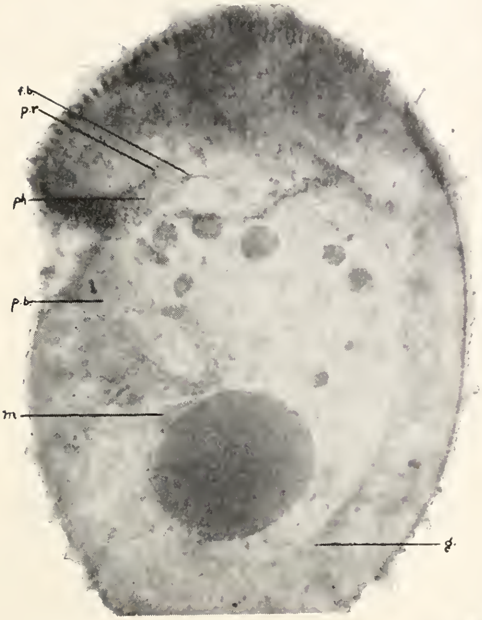
D. Conchophthirius curtus Engl. Dorsal view of organism prepared as in *B.* Note dorsal suture at the extreme posterior end. × 420.

E. Conchophthirius anodontæ Stein. View of an organism somewhat flattened and twisted on its left side. Preparation as in *B.* × 420.

F. Conchophthirius curtus Engl. Dorsal view of a dividing organism to show the formation of the new peristomal regions. Preparation as in *B.* × 300.

G. Conchophthirius curtus Engl. Ventral view of an organism fixed in Schaudinn's fluid and stained with the Borrel stain to show the zone of endoplasmic granules. × 420.

d. s., dorsal suture; *e. z.*, endoplasmic granule zone; *f. b.*, fibrillar bundle; *g.*, gullet; *m.*, micronucleus; *p. b.*, peristomal basket; *p. f.*, post-oral connecting fiber; *ph.*, pharynx; *p. n.*, peristomal net; *p. r.*, pharyngeal ring; *p. z.*, posterior naked zone; *v. s.*, ventral suture.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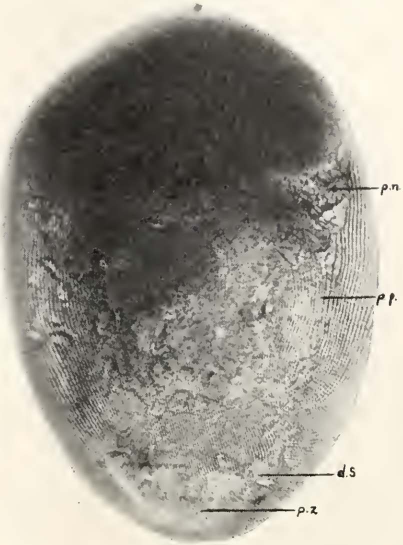
A



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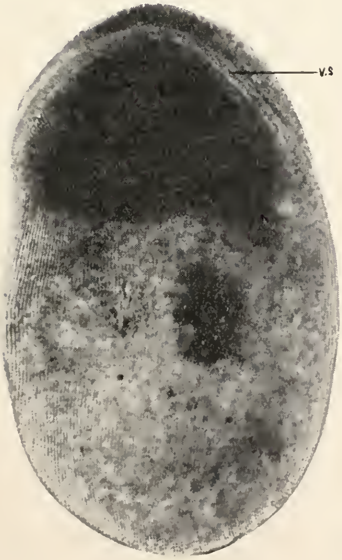

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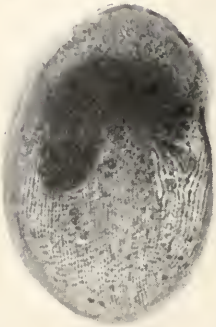

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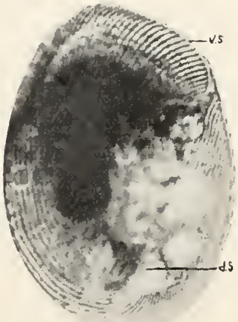

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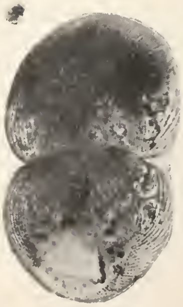

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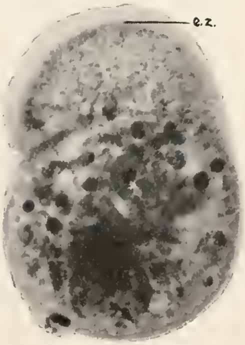

G

(Fig. 3, *exit c. v.*) through which the contents of the vacuole are emptied.

Conchophthirius magna sp. nov. was found in the mantle cavity of about one-fourth of the *Elliptio complanatus* (Dill.) examined in association with *C. anodontæ*. The infestation was never heavy, there being only from ten to twenty organisms in each host. I am a little in doubt as to its purely commensal rôle since its digestive endoplasm is always crowded with epithelial cells and I have never seen any other type of food in the vacuoles. The cells ingested may be sloughed cells but they are regular in outline and stain beautifully.

The Contractile Vacuole

The contractile vacuoles of the three species of *Conchophthirius* described above are identical in every respect but size. The following single description will suffice for all.

A study of the living organism under high magnification reveals the presence of a narrow slit in the dorsal body wall just posterior to the contractile vacuole. This slit is provided with a thickened margin that is highly refractile. During diastole the slit is nearly closed (Fig. 5, *A*) and as the contractile vacuole increases in size there is no apparent change. The vacuole further enlarges and absorbs the accessory vacuoles (Fig. 5, *B*) until its maximum size is reached (Fig. 5, *C*). Very suddenly the slit opens (Fig. 5, *D*), much in the manner of a purse, and the fluid contents of the vacuole pour out. The emptying is not instantaneous but takes about two seconds for completion. In this interval the details of the relationship between the slit and the vacuole can be noted. The edges of the slit are seen to be in contact with the wall of the contractile vacuole and at systole the wall appears to break through at that point.

In material stained with Heidenhain's hæmatoxylin and differentiated in H_2O_2 there is seen to be a network of fine fibers surrounding this slit. The same picture is obtained by the silver method of Klein (Fig. 8, *exit c. v.*). I have no evidence as to the nature of these fibers. That the slit is a permanent structure is demonstrated by its invariable appearance after any good method for showing fibers and also by the modification of the ciliary pattern in this region as seen in Fig. 6, *B* and *D*, and as described by Raabe (1933).

Schuberg (1889) described a tube extending from the contractile vacuole to the dorsal surface. This was also figured by Raabe (1933) and attention directed to the pore. I have never seen this in *Conchophthirius* although I have repeatedly looked for it. All specimens of this group that I have examined have this curious purse-like slit.

MacLennan (1933) has recently given a complete description of the structure of the contractile vacuoles of a number of the Ophryoscolecidæ. In these forms he finds a definite tube leading from the contractile vacuole through the pellicle. The pores at each end of the tube and the tube itself are permanent structures.

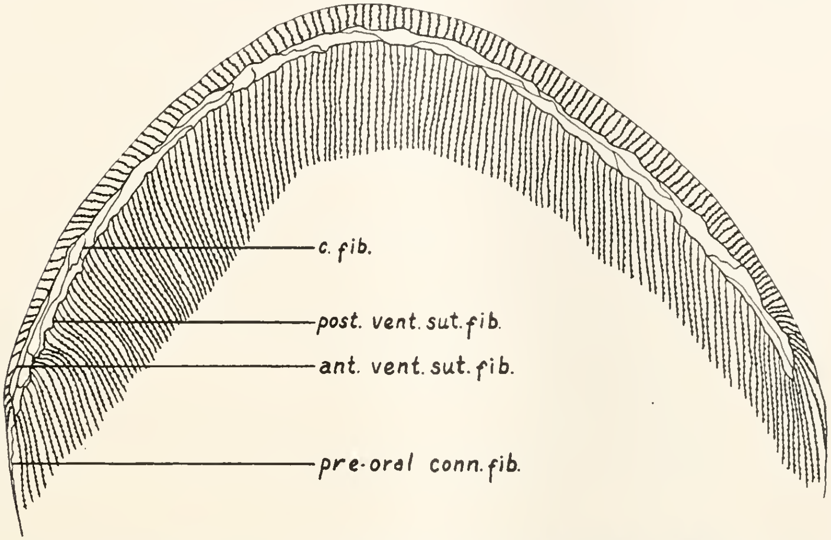

FIG. 7. Ventral view of the anterior end of *Conchophthirius magna* sp. nov. prepared by Klein's method. Note the greater number of ventral ciliary rows than dorsal. × 1000.

ant. vent. sut. fib., anterior ventral suture fiber; *c. fib.*, cross fibers; *post. vent. sut. fib.*, posterior ventral suture fiber; *pre-oral conn. fib.*, pre-oral connecting fiber.

THE NEUROMOTOR SYSTEM

The structures of the neuromotor systems of *Conchophthirius magna, C. anodontæ,* and *C. curtus* are alike with the exception of minor differences of size and extent. For that reason I shall describe the system as seen in *C. magna,* calling attention to points of difference only where they occur in the other two species.

The external fibrillar system can be demonstrated clearly by the silver method of Klein or by the use of Heidenhain's hæmatoxylin differentiated with H_2O_2. After the first method the fibers of the peristomal field are seen quite clearly but their inner connections are not shown. Since the hæmatoxylin method stains both internal and external systems, it is of great advantage to use these two methods in conjunction with one another. When one does so a wonderfully integrated system of connections is seen which I shall refer to as the neuromotor system.

The ventral suture is made up of two irregular fibers, the anterior ventral suture fiber (Fig. 7, *ant. vent. sut. fib.*) and the posterior ventral suture fiber (Fig. 7, *post. vent. sut. fib.*). From the former originate the ciliary rows of the dorsal surface and from the latter the ciliary rows of the ventral surface. These suture fibers come together at their two

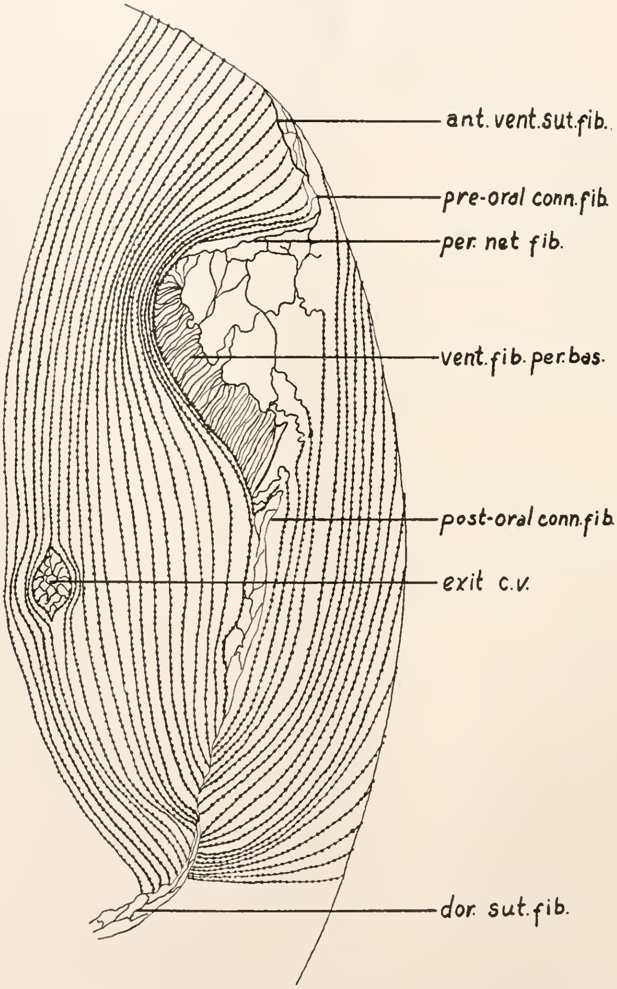

FIG. 8. Dorsal view of the peristomal region of *Conchophthirius magna* sp. nov. prepared by Klein's method. The organism is somewhat flattened. × 1000.

ant. vent. sut. fib., anterior ventral suture fiber; *dor. sut. fib.*, dorsal suture fibers; *exit c. v.*, aperture through which the contractile vacuole empties; *per. net fib.*, peristomal net fiber; *post-oral conn. fib.*, post-oral connecting fiber; *pre-oral conn. fib.*, pre-oral connecting fiber; *vent. fib. per. bas.*, ventral fibers of the peristomal basket. These are probably the same as the "membranella undulans" of *Conchophthirius curtus* as described by Raabe.

ends and are also connected by short, irregularly arranged cross fibers (Fig. 7, *c. fib.*). Near the right border of the organism the joined suture fibers form a connecting fiber that curves up over the edge of the body just posterior to the projection above the cytostome. This is the pre-oral connecting fiber (Figs. 7 and 8, *pre-oral conn. fib.*; Fig. 9, *p. o. c. f.*). It gives rise to two coördinating fibers, one connecting the basal bodies of the dorsal lip ciliary row (Fig. 9, *d. l. c. r.*) and a second short fiber which connects the basal bodies of the pharyngeal ciliary row (Fig. 9, *ph. c. r.*). The pre-oral connecting fiber becomes the peristomal net fiber, which bends sharply to the left in the peristomal field (Fig. 8,

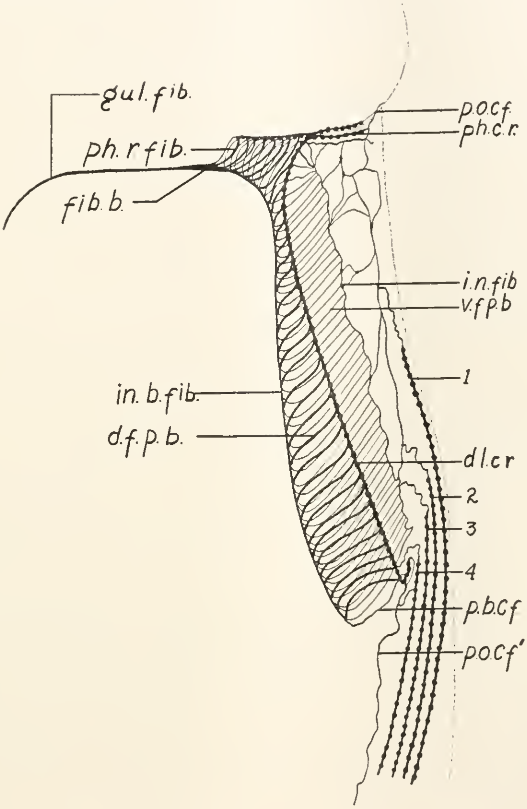

FIG. 9. Diagrammatic representation of the fibrillar system of the peristomal region of *Conchophthirius magna* sp. nov.

d. f. p. b., dorsal fibers of the peristomal basket; *d. l. c. r.*, dorsal lip ciliary row; *fib. b.*, fibrillar bundle; *gul. fib.*, fiber which follows the floor of the gullet; *in. b. fib.*, inner basket fiber; *i. n. fib.*, inner net fiber; *p. b. c. f.*, posterior basket connecting fiber; *ph. c. r.*, pharyngeal ciliary row; *ph. r. fib.*, pharyngeal ring fiber; *p. o. c. f.*, pre-oral connecting fiber; *p. o. c. f'.*, post-oral connecting fiber. *1, 2, 3* and *4*, four short rows of body cilia directly connected to the peristomal net.

per. net fib.). From this fiber originate the secondary fibers of the network which lines the ventral floor of the peristomal field (Fig. 6, *p. n.*). These secondary net fibers, which are very irregular, are bounded on the left by a long inner net fiber (Fig. 9, *i. n. fib.*). From this numerous fine fibers arise and extend inward, lining the ventral side of the peristomal basket (Fig. 6, *A*, *p. b.*; Fig. 8, *vent. fib. per. bas.*; Fig. 9, *v. f. p. b.*). These are the fibers that were called by Raabe (1933) the "membranella undulans" and thought by him to originate from a row of basal bodies. This is indeed the picture one gets with the method of Klein (see Fig. 8) but it is incomplete. The ventral fibers of the peristomal basket bend dorsally and connect at right angles with a long fiber on the floor of the basket, the inner basket fiber (Fig. 9, *in. b. fib.*). From this numerous fine fibers are given off which line the dorsal surface of the peristomal basket (Fig. 9, *d. f. p. b.*) and end in the fiber of the dorsal lip.

The inner basket fiber continues anteriorly along the floor of the pharynx giving off the circular fibers of that region. At the inner end of the pharynx a large fibrous ring is found which encircles the pharynx and fuses with the inner basket fiber (Fig. 6, *A*, *p. r.*; Fig. 9, *fib. b.*). This fusion point is probably comparable to the motorium of *Conchophthirius mytili* (Kidder, 1933*b*) although very much smaller in size. Extending inward from the fibrillar bundle is the long gullet fiber (Fig. 9, *gul. fib.*) which continues throughout the floor of the gullet, finally fraying out at the posterior end.

The inner basket fiber is connected posteriorly with a single peristomal basket connecting fiber (Fig. 9, *p. b. c. f.*) which bends dorsally and to the right and finally connects with the dorsal connecting fiber (Fig. 6, *B*, *p. f.*; Fig. 9, *p. o. c. f.*). This forms the direct connection between the peristomal region and the dorsal suture. There are numerous cross fibers connecting the post-oral fiber with adjacent ciliary rows.

In *Conchophthirius magna* there are four rows of cilia that have their origin in the fibers of the peristomal net (Fig. 9, *1*, *2*, *3* and *4*). These end in the dorsal suture. All the other ciliary rows, with the exception of those of the pharynx and dorsal lip, originate in the ventral suture, and of these all reach the dorsal suture but one, the single row that ends at the slit of the contractile vacuole (Fig. 8, *exit c. v.*).

The conditions in *Conchophthirius anodontæ* and *C. curtus* differ slightly from those of *C. magna* in regard to the ciliary rows about the peristome and the fibers of the ventral and dorsal sutures. The reader is referred to Raabe's (1933) description of these fibers as they all belong to the external fibrillar system.

It will be seen from the above description that all basal bodies are intimately interconnected and also connected with the internal fibrillar system associated with the peristome, pharynx, and gullet.

During fission the old peristome disappears and two new ones are formed. The earliest stages of their formation that I was able to obtain showed a complete set of peristomal fibers, so that direct information as to the origin of these elements is lacking. Later stages (Fig. 6, *F*) show two well-marked, complete peristomes.

Discussion

My measurements of *Conchophthirius anodontæ* and *C. curtus* correspond roughly to those of Raabe (1933), and to those of Engelmann (1862) for the last-named species. Engelmann's sizes for *C. anodontæ* (120 μ to 200 μ) do not agree with mine. Kahl (1931) also states that he has observed specimens of *C. anodontæ* 240 μ long. I think it highly probable that this discrepancy can be explained by the fact that such large forms measured were undoubtedly *C. magna,* as *C. anodontæ* and *C. magna* occur in the same host.

Raabe (1933) has questioned the inclusion of the genus *Conchophthirius* in the Order Trichostomida (Tribus Trichostomata of Kahl, 1931) on the grounds that these organisms possess an undulating membrane in the peristomal region and should therefore be placed in the Order Hymenostomida (Tribus Hymenostomata of Kahl). I have already pointed out that Raabe must have mistaken the fibers of the peristomal basket for an undulating membrane. When observed after the method of Klein these fibers might well be interpreted as cilia arising from the basal bodies of the dorsal lip (see Fig. 8).

The endoplasmic granules described above are so characteristic and so persistent that one is tempted to speculate on their function. While it is perhaps conceivable that they represent secretory granules comparable to those described by Powers (1933*b*) in *Entodiscus borealis,* at present I have no evidence for such an assumption. A discussion of function, therefore, must wait until a future date.

The rhythmic opening and shutting of the purse-like slit dorsal to the contractile vacuole seems to demand a structural explanation. The presence of a network of fibers in this region suggests that they may be of the nature of myonemes, thereby accounting for the action of the slit. It has been tacitly assumed by the majority of protozoölogists that the action of the contractile vacuole was the result of osmotic pressure and that no protoplasmic differentiation was necessary. Ray (1932), however, has described internal fibers about the contractile vacuoles of *Balantidium sushilii,* but he does not state the supposed function of these fibers. I have never found such fibers in the region of the contractile vacuole in *Conchophthirius* but only fine fibers about the slit. Because of the direct connection of these fibers about the slit to the longitudinal fibers of the ciliary rows, I regard them as a part of the external fibrillar system, perhaps functioning as contractile or conductive elements or both.

The concept of the neuromotor system has been discussed by many in the past and more recently by MacLennan and Connell (1931), Powers (1933*b*), Kidder (1933*b*), Turner (1933) and Lund (1933). Lund has given a complete review of the literature dealing with the fibrillar system of *Paramecium* and has correlated the external system (silverline system) with the internal fibrillar system. Turner has demonstrated a similar connection between the two systems in *Euplotes patella*. As has been pointed out by these workers, the conclusion of the American investigators as to the nature and function of these various fibers is largely in agreement. The acceptance of a system of coördinating fibers, functioning as impulse transmitters, seems to be inevitable. This does not exclude the possibility, as mentioned by Powers (1933*b*), that elements connected with this system may have other functions as well, such as supportive and contractile. Indeed, Powers suggests just such a triple function for the " stomatostyle " of *Entodiscus borealis*.

My observations on the various species of *Conchophthirius* have led me to agree entirely with the above conclusions. Also I must point out that the possibility of a set of fibers functioning both as conductile and contractile elements seems to be strengthened by a study of members of this genus. The fibers encircling the pharynx are directly connected with the various peristomal fibers and also with the coördinating fibers of the pharyngeal ciliary row, yet the pharynx is capable of complete contraction. The contraction occurs when food bodies are swept in and by this action the food is forced into the gullet. It seems logical to suppose that the local contraction of the pharynx is brought about by the pharyngeal fibers. The fibers surrounding the slit above the contractile vacuole may be supposed likewise to possess a dual function. It seems possible that in addition to the functions of contractility and conductivity these two sets of fibers may lend some support to their two areas which are subjected to the greatest strains.

No large body could be demonstrated, in the three species described above, that would correspond to the motorium of *Conchophthirius mytili* (Kidder, 1933*b*). This is not a serious issue, and in no way impairs the concept of a neuromotor system. Turner (1933), after carefully re-investigating the fibrillar system in *Euplotes patella*, came to the conclusion that the motorium of Yocom (1918) was only a pellicular fold and that the fibers joined one another directly. The situation described above corresponds to these findings. In the position occupied by the motorium of *Conchophthirius mytili* (under the pharynx), the fibers of *C. anodontæ, C. curtus,* and *C. magna* join in a bundle and instead of having a massive structure only the thickening due to the fusion of fibers is seen. This bundle is the only concentration of material that could be interpreted as a coördinating center, and I see no objection to the idea that it may function as such.

The specific differences that exist between the members of the genus *Conchophthirius* from fresh water mussels involve the pattern of the ciliary rows, the position and extent of the posterior dorsal suture, the position, shape, and number of the nuclei and to some extent the size. In no case is the difference so profound as between *Conchophthirius mytili, C. steenstrupii* or *C. caryoclada* where the relationship of the position of the cytostome to the rest of the body varies greatly. It may be found advisable, when more comparative observations have been made, to subdivide the genus, restricting the term *Conchophthirius* to those forms with the general structural relationships possessed by the type species *Conchophthirius anodontæ.*

SUMMARY

1. Three species of the genus *Conchophthirius* Stein are described: (1) *C. anodontæ* (Ehr.) Stein, from the mantle cavity, palps, and gills of *Elliptio complanatus* (Dill.); (2) *C. curtus* Engelmann, from the mantle cavity of *Anodonta marginata* Say, *A. implicata* Say, *A. cataracta* Say, *Lampsilis radiata* (Say), *L. cariosa* (Say), and *Alasmidonta undulata* (Say); and (3) *C. magna* sp. nov. from the mantle cavity of *Elliptio complanatus* (Dill.).

2. The chief structural differences between the three species are noted in the shape, size, and extent of the peristome, distribution and size of the endoplasmic granules, distribution and number of the ciliary rows, type of posterior dorsal suture, and shape, size, and number of nuclei.

3. The host specificity seems to be marked in the case of *Conchophthirius magna,* less marked in the other two species.

4. Cross infection undoubtedly takes place by swimming trophozoites.

5. A peculiar permanent slit through which the contractile vacuole discharges is described.

6. A well integrated and closely interconnected neuromotor system is decribed, consisting of external, internal, and peristomal fibrillar systems.

7. The neuromotor system is thought to be mainly conductile but some parts of it may possibly be contractile or even supportive.

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Vol. LXVI, No. 2 April, 1934

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

BIOLOGICAL EFFECTS OF HEAVY WATER

E. NEWTON HARVEY

FROM THE PHYSIOLOGICAL LABORATORY, PRINCETON UNIVERSITY

EDITOR'S NOTE:

The second sentence of Conclusion 2, p. 28, of the paper by Roberts Rugh in the February issue should be corrected to read as follows:

". . . The number of eggs that leave the ovary depend, at least in part, on the quantity of pituitary injected."

nitrous acid from the oxyhydrogen flame burning in air. Water so formed is toxic to *Paramœcia*, killing them instantly with discharge of trichocysts, whether it contains 97 per cent heavy water or only 0.2 per cent heavy water, an effect undoubtedly due to its acidity. If redistilled from alkali alone or from alkali and $KMnO_4$, the 0.2 per cent heavy water is harmless while the 97 per cent heavy water kills *Paramœcium* in 6 to 15 hours, without discharge of trichocysts. The 0.2 per cent heavy water formed by oxyhydrogen combustion and redistilled from alkali and permanganate is also non-toxic to *Euglena, Amœba, Epistylis,* luminous bacteria and other small organisms, so that it appears reasonably certain that any effects of concentrated heavy water treated in the

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BIOLOGICAL EFFECTS OF HEAVY WATER

E. NEWTON HARVEY

From the Physiological Laboratory, Princeton University

The observations of Lewis (1933) on the germination of seeds and of Taylor, Swingle, Eyring, and Frost (1933) on various fresh water animals have indicated that concentrated deuterium water or heavy water is toxic for, or at least unfavorably affects living matter. The cause of this action is at present unknown, but certain possibilities can be eliminated by the experiments which are reported in this paper. It is obvious that a mass of information must be gathered before interpretation of results becomes possible, and the following observations are frankly exploratory.

The water used was 95 to 97 per cent heavy water, kindly supplied by Professor H. S. Taylor of the Chemistry Department.

Because of the extreme sensitivity of living organisms to small amounts of impurities of various kinds, great attention was paid to the purification and testing of the samples of heavy water used. In the various stages of preparation of heavy water from electrolysis and burning of the hydrogen and oxygen formed, there is possibility of oxidized hydrocarbons, possibly aldehydes (from ozone acting on rubber tubing), being formed and also readily detectable amounts of nitrous acid from the oxyhydrogen flame burning in air. Water so formed is toxic to *Paramœcia,* killing them instantly with discharge of trichocysts, whether it contains 97 per cent heavy water or only 0.2 per cent heavy water, an effect undoubtedly due to its acidity. If redistilled from alkali alone or from alkali and $KMnO_4$, the 0.2 per cent heavy water is harmless while the 97 per cent heavy water kills *Paramœcium* in 6 to 15 hours, without discharge of trichocysts. The 0.2 per cent heavy water formed by oxyhydrogen combustion and redistilled from alkali and permanganate is also non-toxic to *Euglena, Amœba, Epistylis,* luminous bacteria and other small organisms, so that it appears reasonably certain that any effects of concentrated heavy water treated in the same way must be due to the deuterium it contains and not to unknown impurities. There is always a possibility of doubt that minute traces of some unknown impurity may be present in a minimal effective concentration, but all known precautions have been taken to remove such. In the experiments reported below, heavy water redistilled from alkali has always been used and in most cases heavy water redistilled from alkali and permanganate.

EFFECT ON LUMINESCENCE

The dry powdered luminous organism, *Cypridina*, when added to 97 per cent heavy water luminesces just as brightly and with the same color as in distilled water.

A suspension of luminous bacteria[1] (*Vibrio phosphorescens*, a fresh water form) luminesces about as brightly in approximately 85 per cent heavy water as in distilled water and the light lasts for over 24 hours in each, becoming less and less bright. After five days the bacteria in both heavy and distilled water, when inoculated in culture media in ordinary water, grew and luminesced normally. When inoculated in culture media in ordinary water after 41 days in heavy water, the bacteria also grew and luminesced normally. It is quite evident that the heavy water does not kill these forms.

Experiments with a salt water bacterium, isolated from squid at Woods Hole, Mass., added to heavy water plus salt (about 85 per cent final concentration), showed a marked diminution of luminescence in the 85 per cent heavy water after 4 hours as compared with ordinary water. In another experiment 63 per cent heavy water dimmed in 3 hours, but 36 per cent heavy water did not appreciably. The luminescence of the salt water bacterium is undoubtedly affected in the heavy water.

EFFECT ON GROWTH OF LUMINOUS BACTERIA

The next experiment was devised to find out if luminous bacteria would grow in a culture medium made up in 97 per cent heavy water. Two kinds of bacteria were also used, the fresh water form (*Vibrio phosphorescens*) and the salt water form. The sterile culture medium consisted of: 23 mg. bactonutrient agar, 10 mg. glycerine, 1 mg. $CaCO_3$ per cc. For the salt water form 30 mg. table salt was added, and for the fresh water form 3 mg. table salt. In one experiment with the fresh water form there was some growth and luminescence, while in two other experiments there was some growth but none or very faint luminescence.[2]

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[1] I am deeply indebted to Mr. I. M. Korr for cultures of both forms of luminous bacteria.

[2] Frequently luminous bacteria will grow but will not luminesce under adverse conditions such as treatment with ultra-violet light or high temperatures (38°).

There was good growth and luminescence on the ordinary water controls.

Using the salt water form, in three experiments carried out at different times, a good diffuse[3] luminescent growth occurred in the 94–97 per cent heavy water medium, whereas a bright streak of growth and luminescence appeared on the ordinary water medium. After 24 days bacteria were transplanted from the heavy and ordinary water media to new ordinary water culture media and they grew and luminesced.

The conclusions are that luminous bacteria will grow slowly and one species will luminesce in 97 per cent heavy water, but not as well as in ordinary water, and that they are not killed by long contact with heavy water.

Effect on Protozoa and Small Organisms

Although *Paramœcium* and *Euglena* were studied by Taylor, Swingle, Eyring and Frost (1933), new samples of 95 per cent, 97 per cent and 100 per cent heavy water were tested on these forms and others to see if various samples of heavy water would give the same effects. It was found that they did. The experiments were carried out in hanging drops on cover glasses on depression slides sealed with vaseline. The drop of heavy water was mixed with a minute drop of water containing the organisms. This dilutes the 97 per cent heavy water to an unknown amount, possibly to 85–90 per cent.

Paramœcia almost immediately give the avoiding reaction and swim slowly in the heavy water, soon appearing somewhat bloated, and in two hours the contractile vacuoles are enormously enlarged and fail to empty. The well-known blisters appear at the surface. The animals are dead and disintegrated in less than 24 hours, while controls in distilled water are normal after 5 days.

One experiment was run with 100 per cent heavy water (somewhat diluted by the *Paramœcia*). They were killed in 6 to 10 hours with a similar sequence of events, while they live for days in 0.2 per cent heavy water. *Paramœcia* never recover in ordinary water when once disintegrated.

Amœba dubia and two rotifers, *Monostyla bulba,* and *Philodina roseola,* in heavy water are killed in from 6 to 20 hours. The *Amœbæ* round up, show no movement (and no large contractile vacuole) and look disintegrated in about six hours, whereas in distilled water, the *Amœbæ*, although rounded up at first, are quite normal after 48 hours, as are also the rotifers. The same statement applies also to another large species of *Amœba* and to a *Vorticella*-like infusorian, *Epistylis,* which almost immediately withdraws its peristome, the cilia beat more and more slowly and soon all movement ceases and disintegration occurs. Controls are normal after 48 hours.

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[3] Although a streak inoculation was made.

The conclusion is that a large number of small organisms are killed in 85–90 per cent heavy water and the more rapidly the greater its concentration.

Recovery of Euglenæ

Euglena gracilis in 97 per cent (slightly diluted) heavy water mostly rounds up and remains immobile for days. On the ninth day, the heavy water was replaced by distilled water and most of the *Euglenæ* recovered and swam about perfectly normally.

Euglena proxima[4] also shows the avoiding reaction, (euglenoid shapes), mostly round up and become immobile in heavy (90 per cent) water, but a certain percentage may be motile for three days although not nearly as active nor as elongate as the controls in ordinary water or water containing 0.2 per cent D_2O. After three days movement in practically all the organisms has ceased. After five days ordinary water was added to greatly dilute the heavy water and most of the *Euglenæ* recovered completely.

The conclusion is that *Euglenæ* are not irreversibly injured by the heavy water.

Effect on Protoplasmic Rotation

One-half of an *Elodea* leaf, showing marked protoplasmic rotation, was placed in 95 per cent heavy water at 25° C. A slowing of rotation occurs at first which very soon begins again, and in 15 minutes rotation is nearly as marked as in the half control leaf in ordinary distilled water at 25°. The heavy water was then replaced by more 95 per cent heavy water and this was again replaced after two hours so that the leaf must finally have been exposed to approximately 95 per cent heavy water with no dilution. The rotation gradually slows over a period of hours and there is still some slow rotation in some cells after 5 hours but much less than in the control. After 24 hours there was still some slow rotation in the leaf in heavy water but practically none in the control in distilled water, but rotation began again later. Another experiment gave a similar result. The cells look quite normal in appearance.

The conclusion is that a gradual slowing of protoplasmic rotation occurs in concentrated heavy water but that it is not marked or rapid and that *Elodea* cells may show rotation and are not injured by heavy water after 24 hours.

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[4] Kindly supplied in pure culture by Dr. R. Glaser of the Rockefeller Institute, Princeton, New Jersey.

Penetration of Heavy Water into Cells

In order to test penetration in a rough way, *Elodea* leaf cells were plasmolyzed in m/2 cane sugar solution and then returned to heavy water, when the protoplasts reëxpand again. The heavy water must have entered the *Elodea* cells to again restore osmotic equilibrium. This method is not sufficient to detect differences in rate of entrance as compared with ordinary water, which should be carried out on the swelling of spherical cells such as *Arbacia* eggs in a hypotonic medium. Effects of heavy water cannot be attributed to non-penetration. Indeed the enormous enlargement of the contractile vacuoles in *Paramœcium* in heavy water points to an interference with the water-eliminating mechanism rather than an influence on water penetration. However, such an interference may be brought about by many unfavorable conditions.

Does H_2O_2 Accumulate in Heavy Water?

In view of observations of Taylor and Pace[5] that heavy water retards the action of liver catalase on H_2O_2, experiments were carried out to determine if the effect of heavy water is so to retard the action of catalase in cells as to cause possible accumulation of H_2O_2 as a result of respiration or possibly of photosynthesis. In either case the destructive effect might be due to accumulation of H_2O_2.

This theory can be tested in three ways. First, by finding if heavy water is toxic for anærobic forms that contain no catalase. Second, by testing ærobic forms, which can also live under anerobic conditions for some time, in absence of oxygen. Third, by testing green organisms like *Euglena* in light and in darkness.

Euglena proxima or *E. gracilis* was placed in 85–90 per cent heavy water on slides in hanging drops and kept both in darkness and in light. Since this concentration of heavy water does not immediately affect all the organisms but merely causes many of them to become spherical and immobile, while others move about slowly, any additional H_2O_2, possibly accumulating in light from photosynthetic processes, might be sufficient to cause a difference in behavior of the organism in light and in darkness. However, no significant difference could be found, although many experiments were performed. In heavy water spherical and immobile *Euglenæ* do not become active in the dark. After three days all the *Euglenæ* were immobile and spherical whether they had been kept in light or in darkness. In general, *Euglenæ* are more active in the dark, distributing themselves uniformly through the drop, whereas in the light they collect in one spot, but this is a light reaction. It occurs in ordinary water also and cannot be connected with the possible accumulation of H_2O_2.

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[5] Private communication from Professor H. S. Taylor.

Paramœcium cannot be tested in heavy water in a pure hydrogen atmosphere because *Paramœcium* in culture medium will not withstand absence of oxygen for any length of time. They are mostly killed in one hour in a hanging culture drop in a pure hydrogen atmosphere.

However, if *Paramœcia* are killed by the accumulation of H_2O_2 in heavy water, the effects of H_2O_2 on *Paramœcia* should be the same as the effects of heavy water. To test this, Merck's superoxol (a very pure preparation of H_2O_2) was added to culture medium containing *Paramœcium* in a concentration which caused disruption and surface blister formation in 1 to 1.5 hours. Only a slight enlargement of the contractile vacuole occurred, but not the enormous enlargement characteristic of a heavy water effect. This may be taken as additional evidence against the view that accumulating H_2O_2 is responsible for the toxic effects on *Paramœcium*.

SUMMARY

Heavy water (85–95 per cent) has been found not to prevent the luminescence of dried *Cypridina* nor to affect the luminescence of a fresh water luminous bacterium but to diminish the luminescence of a marine form; to retard growth of luminous bacteria, sometimes allowing slow growth without luminescence; to kill a number of protozoa and rotifers, but not to kill bacteria and not to injure *Euglena* irreversibly; to affect *Euglena* equally in light and in the dark; to affect only slowly protoplasmic rotation of *Elodea* cells and to penetrate into *Elodea* cells. In view of the slow and often reversible effects of heavy water, its action may be likened to that of a generally unfavorable environment, leading to progressive changes in the cell. No more can be said at present than to suggest that these changes are the result of differential effects on the rate of biochemical reactions which ordinarily proceed at a certain definite rate in relation to each other.

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THE EFFECTS OF LAND DRAINAGE UPON THE EXCESS BASES OF SEA WATER[1]

PHILIP H. MITCHELL AND JULIAN L. SOLINGER

(From the Arnold Biological Laboratory of Brown University and the Woods Hole Oceanographic Institution)

The total titratable alkalinity of sea water, that is, its buffer capacity for acids, tends to reach an equilibrium such that the total buffer capacity divided by the chlorinity is a constant. This fact has been established by several investigators working on samples from the North Sea and its vicinity and by Thompson and Bonnar (1931), who studied the waters of the region of the San Juan archipelago and Puget Sound. The results of Mitchell and Rakestraw (1933) on Atlantic waters of the Cape Cod and Bermuda regions also revealed the tendency toward constancy of ratio. It is to be expected that when the only important change in the sea water composition is that due to the addition or withdrawal of water by rain or evaporation, this and similar ratios should be constant.

It appears, however, that the ratio as observed in different regions is not necessarily the same. The differences might be due, in part at least, to variations in methods of measurement but this appears not to be the entire explanation (Mitchell and Rakestraw, 1933). It would seem, then, that the oceans are not in apparent equilibrium as regards the ratio of excess base to the salts. But although some approach to a constant buffer capacity-chlorinity ratio is discernible in ocean waters, such is not the case, generally speaking, in the waters of sounds, bays and inlets where equilibrium conditions are more actively disturbed.

Thompson and Bonnar have discussed factors which alter the ratio. Among them are photosynthesis and other effects of plant life and of animal life and the effect of land drainage. Our own experience has shown the latter to be a very significant factor tending, as Thompson and Bonnar found, to raise the buffer capacity-chlorinity ratio but showing, in our observations, great variability with locality, character of the soil and rapidity of drainage, that is, whether surface or deep drainage. It has even appeared possible to us that the character of the soil in different regions of the earth might vary sufficiently in potentiality for yielding bases to the sea to account in part for the differences in the ratio as apparently found in different ocean areas.

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[1] Contribution No. 42 from the Woods Hole Oceanographic Institution.

A satisfactory illustration of the land drainage effect was obtained by observations on Narragansett Bay. It is a relatively long and narrow body of salt water. No large, long rivers enter it so that the drainage which it receives is of comparatively local origin. Measurements of buffer capacity were made by a method described in a previous communication (Mitchell and Rakestraw, 1933). The results of typical observations, as shown in Table I, reveal what is probably demonstrable for any similar body of water, namely, that the lower the salinity the higher, in general, is the buffer capacity-chlorinity ratio. This is a tendency rather than an exact relationship because of the disturbing effects of tidal currents, storms, freshets and local conditions such as the relative abundance of plant and animal life.

TABLE I

The Relative Buffer Capacity of Narragansett Bay Water Samples Arranged in Decreasing Order of Chlorinity

Chlorinity *grams per liter*	Ratio $\frac{BC}{Cl}$
19.50	0.1143
19.00	0.1153
18.10	0.1222
17.90	0.1227
17.38	0.1210
17.38	0.1217
17.02	0.1208
17.02	0.1208
16.85	0.1189
16.84	0.1171
16.73	0.1186
16.72	0.1239
16.55	0.1200
16.48	0.1223
16.36	0.1214
16.31	0.1251
16.20	0.1258
16.16	0.1237
16.05	0.1188
15.94	0.1272
15.93	0.1242
15.50	0.1191
15.50	0.1216
15.40	0.1275
15.22	0.1266
15.05	0.1307
14.96	0.1229
14.60	0.1283
14.52	0.1383
14.45	0.1338
14.06	0.1345
13.88	0.1364
13.15	0.1258
13.04	0.1425
11.92	0.1535
5.40	0.1630
3.61	0.1690

In order to demonstrate more clearly the relationship between dilution of estuary waters and their buffer capacities, observations were made upon various series of mixtures of salt water with fresh water from selected sources. In every case progressive dilution caused a rise in the buffer capacity-chlorinity ratio such that the latter plotted as ordinates against the chlorinity as abscissas gave a smooth curve. The rise in the ratio with addition of fresh water was not, however, the same in all cases. To illustrate typical variations, a number of the curves obtained are shown in Figs. 1 and 2.

Curves *A, B* and *C* were obtained by dilution of Narragansett Bay water with that of the Pawtuxet River which drains the watershed supplying the city of Providence. The fact that observations on the undiluted salt water from the bay, giving the lowest point of each curve, do not yield closely approximating results is due to the circumstance that water collections were made at different stages of the tide and in one case (*A*) shortly after an easterly storm which raised the salinity of the water above its usual value. This is in part the explanation of the different levels of the curves. But it may also be seen that the slopes of the curves are different.

Curve *A*, obtained on March 7, 1933, illustrates the effect of an easterly storm in that the undiluted bay water has a relatively high salinity and a low ratio. The rise in the ratio with progressive dilution is an intermediate one increasing from 0.1253 (Cl, 14) to 0.1583 (Cl, 4).

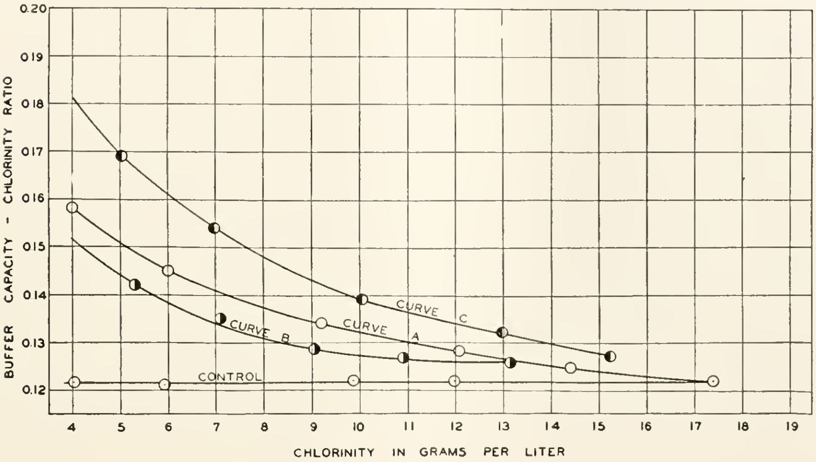

FIG. 1. Effect of successive dilutions of Narragansett Bay water with Pawtuxet River water. Curve *A*, in March, Curve *B*, after a freshet in April, Curve *C*, in June. For the control, dilutions were made with distilled water.

Curve *B*, obtained on April 11, 1933, illustrates the effect of mere surface drainage. There had been a light snowfall followed by especially heavy and prolonged rain. The river was at spring freshet level. The ratio increased only from 0.1252 (Cl, 14) to 0.1520 (Cl, 4).

Curve *C*, however, illustrates the more typical effect of the true soil drainage of this area. Sampled on June 13, 1933, after a period of approximately normal rainfall, the river water raised the ratio from 0.1293 (Cl, 14) to 0.1820 (Cl, 4).

There is then a seasonal variation in land drainage effects upon the

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titratable base of estuary waters. The larger the proportion of deep soil drainage to surface run-off, the greater is the effect in this particular type of drainage area.

Curves *D* and *E* illustrate the relatively large effect of deep soil drainage in another way. The salt water in these cases came from Sakonnet Point at the more easterly of the two mouths of Narragansett Bay. The fresh water in the case of curve *D* was taken from a pond about one mile inland from the Sakonnet shore and in the case of curve *E* from a clear deep spring near the edge of the same pond. The ratio in curve *D* increased from 0.1240 (Cl, 16) to 0.1381 (Cl, 6) while in curve *E* the increase was much greater, from 0.1182 (Cl, 16) to 0.1490 (Cl, 6).

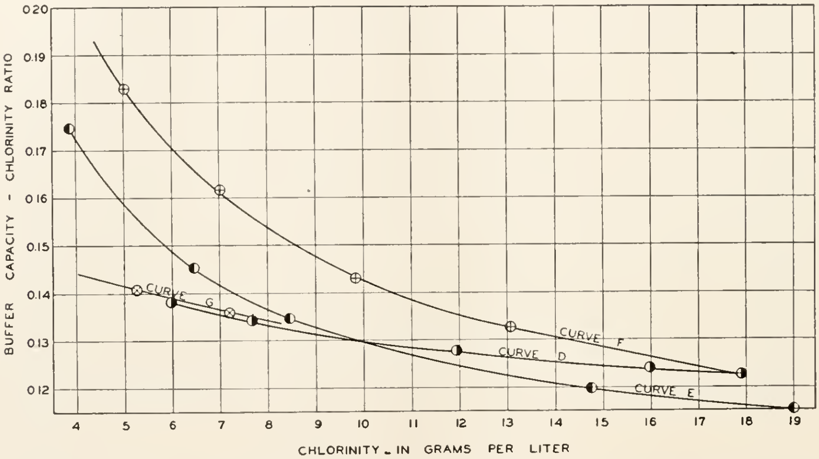

FIG. 2. Effect of successive dilutions of sea water with fresh water from various sources. Curve *D*, with pond water, Curve *E*, with spring water, Curve *F*, with pond water from swampy soil, Curve *G*, with pond water from sandy soil.

Another factor influencing the drainage effect is the character of the soil. Curves *F* and *G* illustrate this fact. The sea water used for both of these sets of measurements was taken from Woods Hole Harbor on June 14, 1933, after a period of only moderate rainfall. The fresh water used for the dilutions of curve *F* was collected on the same day from a nearby pond in a swampy area, while that used for curve *G* came from another pond about four miles away entirely surrounded by very sandy soil. Curve *F* shows a rise of the ratio to 0.1832 (Cl, 5) but curve *G* rises only to a ratio of 0.1416 at the same dilution. Apparently a barren, sandy soil yields comparatively little excess base to its drainage water.

The curve at the bottom of Fig. 1 indicates data for a control upon the accuracy of the measurements and the reproducibility of the results. It was obtained by adding distilled water to a sample of Narragansett Bay water. It shows no change, in excess of the limits of observational error, in the ratio even when the chlorinity is diluted to 4 grams per liter. The probable error of a single determination of the ratio is found to be approximately ± 0.0007, a value in accord with numerous other estimations of the probable error of this method.

Summary

1. Land drainage tends, in general, to raise the buffer capacity-chlorinity ratio of sea water.
2. Heavy rains measurably decrease the proportional effect.
3. The effects of deep soil drainage are greater in raising the ratio than are those of mere surface drainage.
4. The character of the soil alters the effect. Sandy soil may yield measurably less base than a richer soil.

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PARADOX PHENOMENA IN THE CARDIAC GANGLION OF LIMULUS POLYPHEMUS [1]

IPING CHAO [2]

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It was found by Zwaardemaker (1917, 1925) that when a frog's heart is perfused with a Ringer's solution containing 40 mgm. thorium nitrate per liter instead of the 170 mgm. potassium chloride normally present, a temporary arrest of the beat occurs at the moment of replacing the thorium-containing by the potassium-containing Ringer's solution. This temporary inhibition of the activity of the heart on replacing a light radioactive element (potassium and rubidium in one group) by a heavy radioactive element (uranium, thorium, radium, emanation and ionium in another group), or vice versa, was called by Zwaardemaker the radio-physiologic paradox. Shortly afterwards Libbrecht (1920) found that the same temporary inhibition of the frog's heart can be obtained on change from a K-free to a normal Ringer's solution, and he called this striking phenomenon the potassium-paradox. Libbrecht (1921) also described a thermo-paradox in the frog's heart on change from a warm to a cold Ringer's solution. More recently Kisch (1930) reported a Ca-paradox on change from a Ca-free to a normal Ringer's solution and a Sr-paradox on change from a Ca-free to a normal Ringer's solution containing an equivalent amount of $SrCl_2$ instead of $CaCl_2$.

Paradoxes of this kind are found not only in the frog's heart but also in the hearts of other animals and in other tissues. Thus the K-paradox has been described in the rabbit's heart by Busquet (1921, 1922*b*) and in the heart of the spider crab by Wells (1928). Busquet (1922*a*) also observed a similar paradox in the rabbit's heart on change from a K-free Ringer's solution to the same solution containing ammonium chloride. The K-paradox has been demonstrated in the automatic movement of the frog's œsophagus by Bakker (1927) and in the isolated fore-gut of insect by Hobson (1928). It is also found in the ganglion of the *Limulus* heart (Chao, 1933). In this paper a further study of the conditions for the production of the K-paradox in this ganglion will be presented, together with observations on the Ca-paradox and thermo-paradox.

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[1] A preliminary report of this work appeared in the *Collecting Net,* 1933, **8**: 341.

[2] Partly aided by a Collecting Net Scholarship.

The experimental procedure has been described in a previous paper (Chao, 1933). Briefly, the ganglion is isolated from the heart musculature posteriorly and the heart is sectioned behind the second segment, leaving the ganglion in connection with the anterior two segments of the heart only. The heart is mounted in sea water for graphic registration of the contractions, which result from impulses originating in the nerve cells of the ganglion; the latter is immersed in a Ringer's solution. The Ringer's solution contains 445 millimols NaCl, 8.9 millimols KCl and 37 millimols $CaCl_2$ in a liter. It can be rapidly replaced by another solution without disturbing the ganglion-heart preparation. All experiments (except those on the thermo-paradox) are performed at the room temperature; this fluctuates usually within one degree during an experimental period of two to three hours, and varies between 22° to 26° C. during the whole season.

THE POTASSIUM-PARADOX

When a ganglion is immersed in a K-free Ringer's solution, the rate is slightly increased. Ten minutes later the K-free Ringer's solution is replaced by a normal Ringer's solution;[3] the rate and amplitude, instead of returning gradually to normal, are immediately decreased, and in some cases the heart-beat may be even completely arrested. This temporary inhibition is the characteristic phenomenon of the paradox; it lasts for a short period and is followed by a gradual recovery. The extent of the inhibition depends upon several factors, which will be discussed in order.

The Effect of Repeated Immersion in the K-free Ringer's Solution

In general, the K-paradox is more readily obtained on a second or third repetition than on the first immersion of a fresh ganglion in the K-free Ringer's solution. The following experiment (No. 86), although an extreme case, illustrates this point particularly well. When this ganglion was first immersed in a K-free Ringer's solution for ten minutes, no K-paradox was obtained on return to the normal Ringer's solution. Twenty minutes later the ganglion was again immersed in a K-free Ringer's solution for another ten minutes. Then on change of solutions the rate decreased from 15.5 beats per minute (the rate at the tenth minute in the K-free Ringer's solution) to 5, 6, 6, 8, 8, 8, 8, 9, 9, and 9 in successive minutes in the normal Ringer's solution; after twenty minutes the rate was 9.5. Corresponding to the primary decrease in rate, there was also a slight decrease in amplitude (Fig. 1, *a*). These changes in rate and amplitude were even more pronounced after the third immersion in the K-free Ringer's solution for the same length of time. On transfer to the normal Ringer's solution the heart beat was immediately arrested for a minute and a half (Fig. 1, *b*).

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[3] The K-paradox can also be demonstrated on changing from a K-free Ringer's solution to sea water.

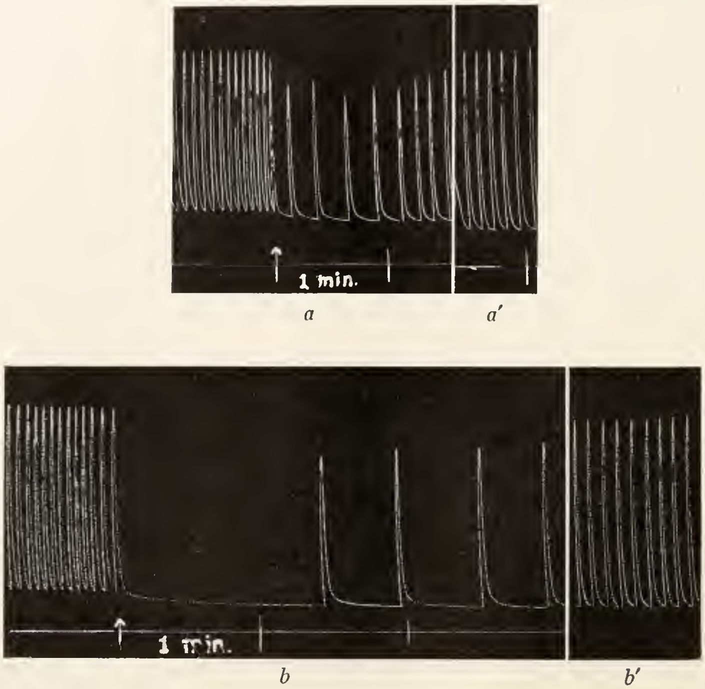

FIG. 1. Experiment No. 86. Effect of repeated immersion in the K-free Ringer's solution on the production of the K-paradox. (*a*) After a second immersion in a K-free Ringer's solution for ten minutes the ganglion, which did not give any sign of the K-paradox on the first trial, was returned to a normal Ringer's solution at the point marked by an arrow. (*a′*) Sixteen minutes later in the normal Ringer's solution. (*b*) K-paradox obtained on a similar immersion for the third time. (*b′*) Twelve minutes later in the normal Ringer's solution.

All figures read from left to right, and the change of solution is always marked by an arrow.

In order to evaluate quantitatively the paradoxical effects of these two trials, the average rate for the first five minutes immediately following the change from the K-free to the normal Ringer's solution (R') was compared with the final rate in the latter (R) when recovery is complete. Expressed as the average percentage decrease in rate for the first five minutes following the change of solutions $[(R - R')/R \times 100]$, the results of these trials are given in Table I.

TABLE I

Average percentage decrease in rate for the first five minutes on return to the normal Ringer's solution after repeated immersions in the K-free Ringer's solution. (Experiment No. 86.)

Number of trials	Percentage decrease in rate
First	0
Second	31
Third	74

In this manner the intensity of the K-paradox is conveniently expressed in any experiment in terms of the primary decrease in rate. The greater the proportional decrease in rate, the more pronounced is the paradoxical effect.

Since the paradoxical effect is increased with the repeated immersion in the K-free Ringer's solution, a difficulty arises in comparing instances of K-paradox obtained under different conditions, even when the same ganglion is used. The difficulty may be obviated by performing the experiments in pairs, the order of the successive trials being reversed in the two or three trials of each pair.

TABLE II

Average percentage decrease in rate for the first five minutes on return of the ganglion to normal Ringer's solution after immersions of unequal durations in K-free Ringer's solution.

Duration of immersion in the K-free solution	Decrease in rate			Average
	Exper. No. 9	Exper. No. 10	Exper. No. 11	
minutes	*per cent*	*per cent*	*per cent*	
10	7.5	20	28	19
20	23	—	31	27
30	35	39	—	37

The Effect of the Duration of Immersion in the K-free Ringer's Solution

A certain minimum duration of immersion in the K-free Ringer's solution is necessary for the production of the K-paradox. This minimum duration, however, is difficult to determine with exactitude for the above-mentioned reason. On the other hand, if the exposure to the

K-free Ringer's solution is too long, so as to cause irreversible change in the ganglion, the rate and amplitude may continue to decrease on return to the normal Ringer's solution and no recovery will be seen. Within certain limits, up to an immersion of about thirty minutes, the changes are perfectly reversible in a good ganglion. Table II gives the comparison of the K-paradox obtained after immersions of 10, 20, and 30 minutes in the K-free Ringer's solution for three different ganglia.

In experiment No. 9 the order of experimentation was from shorter to longer durations; in experiments Nos. 10 and 11, from longer to shorter durations. As the duration of immersion in the K-free Ringer's solution increases, the decrease in rate becomes greater, and the primary decrease in amplitude is more pronounced; also the time for complete recovery is more prolonged.

The Effect of the Difference in K-content in the Two Solutions

Between the K-free and the normal Ringer's solution the difference of the K-content is about 9 millimols per liter, and the K-paradox usually appears on transfer to the normal Ringer's solution after an immersion of 10 minutes in the K-free solution. (In most of the experiments an immersion of ten minutes was adopted as the standard duration.) The paradox is also seen when a ganglion is transferred from the K-free solution to one containing an excess of KCl (*e.g.*, 18 millimols per liter). Although the excess of KCl by itself produces a rapid rhythm, yet the primary inhibition, particularly of the amplitude, is still obvious. In general, the paradoxical effect obtained on change from the K-free Ringer's solution increases, within certain limits, with the K-content of the second solution.[4]

When a K-poor Ringer's solution is used (*e.g.*, one containing 1 or 2 millimols KCl per liter) the K-paradox is still obtained on change to a normal Ringer's solution, or to one containing an excess of KCl. It is also obtained on change from a normal Ringer's solution to a Ringer's solution containing a large excess of KCl, about 70 to 100 millimols per liter. In general, a relatively great difference in the K-content of the two solutions is necessary for the production of the K-paradox, when the change is from a normal Ringer's solution to one containing an excess of KCl. The paradoxical effect is thus not directly proportional to the difference in the K-content. It is important to note that no such paradoxical effect is ever observed when the K-content is changed in the *decreasing* direction (*e.g.*, change from a K-excess to a normal Ringer's solution, or from a normal to a K-poor Ringer's solution). The determining factor for the K-paradox is always the *sudden increase* in the K-content.

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[4] The K-paradox can also be observed on change to a Ringer's solution containing only 1 millimol KCl per liter after an immersion in the K-free Ringer's solution for twenty minutes.

The Effect of the Presence of Other Electrolytes

When a fresh ganglion is immersed in a pure isotonic sucrose solution the rhythm can be maintained quite well for a considerable period up to half an hour; the precise duration depending upon the condition of the ganglion. If the ganglion thus active in pure sucrose solution is transferred to a sucrose solution containing KCl in the concentration normally present in the Ringer's solution, it is found that both the rate and the amplitude of the beat undergo a continuous decrease and the rhythm is gradually arrested in a few minutes; no K-paradox can be seen. This observation indicates that the presence of electrolytes other than KCl, either in the K-free or in the K-containing solution or in both, is necessary for the production of the K-paradox. There are thus two electrolytes ($CaCl_2$ and KCl) and two solutions (the K-free and the K-containing solutions) to be considered.

Is $CaCl_2$ Necessary for the Production of the K-paradox?—The K-paradox can be readily obtained on change from a pure isotonic NaCl solution to a normal Ringer's solution. Calcium is thus not necessary in the K-free solution. It is also not necessary in the K-containing solution, for the K-paradox is obtained on transfer from a K-free to a Ca-free Ringer's solution. The K-paradox appears on change from a pure isotonic NaCl solution to a Ca-free Ringer's solution (Fig. 2).

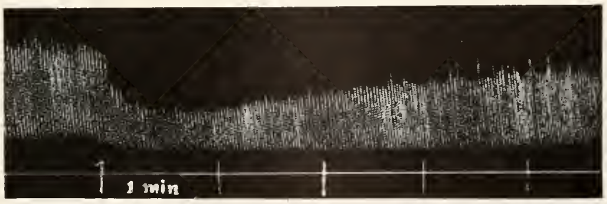

FIG. 2. Experiment No. 79. K-paradox obtained on change from a pure isotonic NaCl solution to the same containing 9 millimols KCl per liter (*i.e.*, the Ca-free Ringer's solution).

This last observation shows conclusively that $CaCl_2$ is not necessary for the production of the K-paradox and can be omitted from both the K-free and the K-containing solutions.

It should be noted, however, that in the last experiment cited, where the K-paradox appears on change from a pure NaCl solution to a Ca-free Ringer's solution, the rate is only slightly inhibited for a short period and the primary decrease in amplitude may not appear at all. The difficulty with which the K-paradox is obtained under these circumstances is due to the fact that both the pure NaCl and the Ca-free solutions normally give rise to a rapid and irregular rhythm.

On the other hand, when an excess of $CaCl_2$ (*e.g.*, 100 millimols per liter) is present in the K-free solution, the K-paradox can still be obtained on return to the normal Ringer's solution if the rate and amplitude are not unduly decreased while the ganglion is in the former solution. Similarly, the K-paradox still can be obtained on change from a K-free Ringer's solution to a normal Ringer's solution containing an excess of $CaCl_2$ (100 millimols per liter), provided the $CaCl_2$ does not reach such a concentration as to inhibit the rhythm by itself (Fig. 3). Finally the K-paradox has been obtained on change from a K-free to a K-containing Ringer's solution, both containing the same excess of $CaCl_2$ (80 millimols per liter). It seems, therefore, that the K-paradox is not abolished by the removal of $CaCl_2$, nor is it antagonized by the excess of $CaCl_2$, and that the general effect of $CaCl_2$ is only secondary, consisting in balancing part of the toxic action of NaCl.

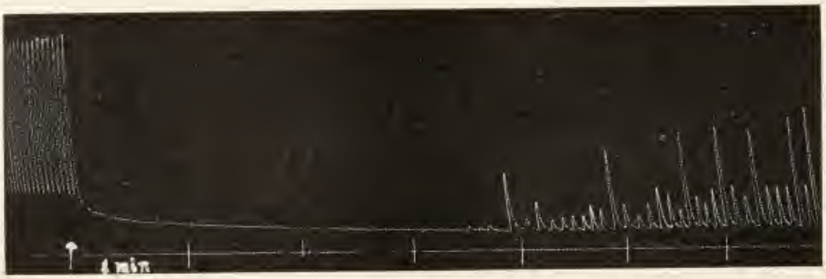

FIG. 3. Experiment No. 92. K-paradox obtained on change from a K-free Ringer's solution to a normal Ringer's solution containing an excess of $CaCl_2$ (100 millimols per liter).

Is NaCl Necessary for the Production of the K-paradox?—Since the presence of some other electrolyte either in the K-free or in the K-containing solution has been shown to be necessary for the production of the K-paradox, and since $CaCl_2$ has been shown to be unnecessary, the logical conclusion follows that NaCl must be the necessary electrolyte. In fact, the paradoxical effect is found to be progressively diminished with progressive decrease in the NaCl-content of the K-free solution. When a fresh ganglion is immersed in a mixture of 30 volumes of a K-free Ringer's solution[5] and 70 volumes of isotonic sucrose solution, hardly any sign of the K-paradox can be demonstrated on return to the normal Ringer's solution. The K-paradox is obtained only on a second or third trial with such a solution, and the paradoxical effect is always less marked than that obtained with a 100 per cent K-free Ringer's solution. Table III gives the results of the primary decrease in rate for four experiments. In these experiments the first trials with either the 100 per cent or the 30 per cent K-free Ringer's solution were discarded, and in two experiments the order of treatment with the two solutions was reversed for the purpose of control, as already described.

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[5] K-free Ringer's solution is used instead of pure NaCl solution on account of the less toxic nature of the former.

When NaCl is entirely replaced by sucrose in the K-free solution the K-paradox is entirely abolished. No paradoxical effect has ever been observed on change from a pure isotonic sucrose solution, or from the same solution containing 37 millimols $CaCl_2$ in a liter, to the normal Ringer's solution.

TABLE III

Average percentage decrease in rate for the first five minutes on change to normal Ringer's solution after an immersion of ten minutes in (1) a 100 per cent and (2) a 30 percent K-free Ringer's solution.

Experiment No.	Decrease in 100 per cent solution *per cent*	Decrease in 30 per cent solution *per cent*
75	36	29
76	36	20
77	33	18
78	54	16
Average	40	21

NaCl, however, is necessary only in the K-free Ringer's solution, for the K-paradox can be obtained on change from a pure isotonic NaCl

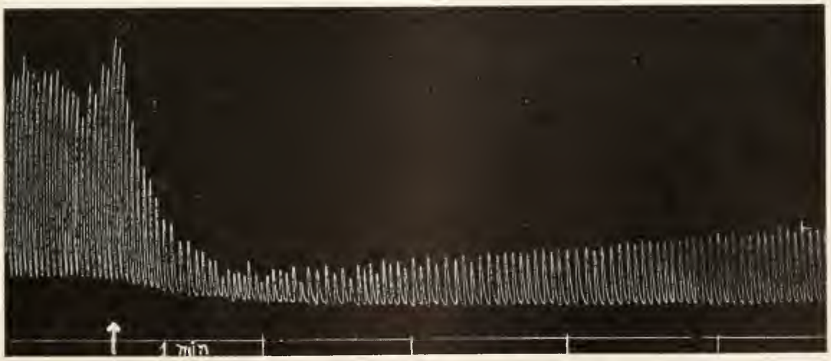

FIG. 4. Experiment No. 63. K-paradox obtained on change from a pure isotonic NaCl solution to a sucrose solution containing 9 millimols KCl per liter.

solution to a sucrose solution containing KCl in the concentration normally present in Ringer's solution[6] (Fig. 4).

The Calcium-paradox

When a ganglion is immersed in a Ca-free Ringer's solution for several minutes the rate of the heart-beat is accelerated considerably. If then the Ca-free Ringer's solution is replaced by a normal Ringer's solution, the rate falls progressively to normal (rapidly at first and more gradually later), while the amplitude undergoes a somewhat rapid decrease to a minimum height followed by a period of gradual rise to normal (Fig. 5). This primary and temporary decrease in amplitude on change from a Ca-free to a normal Ringer's solution resembles that observed in the K-paradox, though it is less pronounced, and is known as the Ca-paradox. Like the K-paradox, it is more readily obtained on a second or a third repetition than on the first immersion of a fresh ganglion in the Ca-free Ringer's solution; and the primary decrease in amplitude is greater after a longer period of immersion in the Ca-free Ringer's solution. The change illustrated in Fig. 5, for instance, was obtained after an immersion of a fresh ganglion in the Ca-free Ringer's solution for twenty minutes; a second immersion for ten minutes gave a very small primary decrease in amplitude indeed.

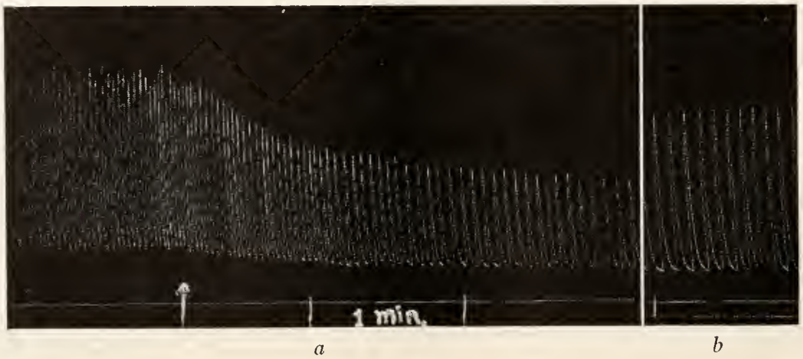

FIG. 5. Experiment No. 26. (*a*) Ca-paradox obtained on change from a Ca-free to a normal Ringer's solution after the ganglion has been immersed in the former for twenty minutes. (*b*) Eighteen minutes later in the normal Ringer's solution.

The Ca-paradox differs from the K-paradox essentially in two respects. In the first place, the primary temporary inhibition affects the amplitude only; during the process of recovery, the rate returns progressively to normal without first decreasing to a minimum. Since the amplitude is not always a good criterion for quantitative evaluation of the changes (*e.g.*, the amplitude is often markedly irregular in such a solution), and since the Ca-free Ringer's solution is more toxic than the K-free Ringer's solution, the Ca-paradox is not so easily obtained as the K-paradox. For this reason the observations on the Ca-paradox have been relatively few. In the second place, it seems that the Ca-paradox is obtained only on change from a Ca-free to a normal Ringer's solution. It is not obtained on change from a Ca-free Ringer's solution to a sucrose solution containing 37 millimols $CaCl_2$ in a liter, or to a K-free Ringer's solution.[7] Furthermore, the presence of an excess of $CaCl_2$ in the second solution prevents the appearance of any change of the paradoxical kind; there is simply a progressive decrease in rate and amplitude. In short, the Ca-paradox is not proportional to the difference in the Ca-content of the two solutions and requires the presence of both NaCl and KCl.

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[6] The K-paradox can be more readily demonstrated on change from a K-free Ringer's solution to a sucrose solution containing KCl.

The Thermo-paradox

The rate of the heart-beat of *Limulus* is always increased at a higher temperature and decreased at a lower temperature. Therefore, on suddenly bringing a ganglion from a Ringer's solution at the room temperature to a warm Ringer's solution, the rate is almost instantaneously in-

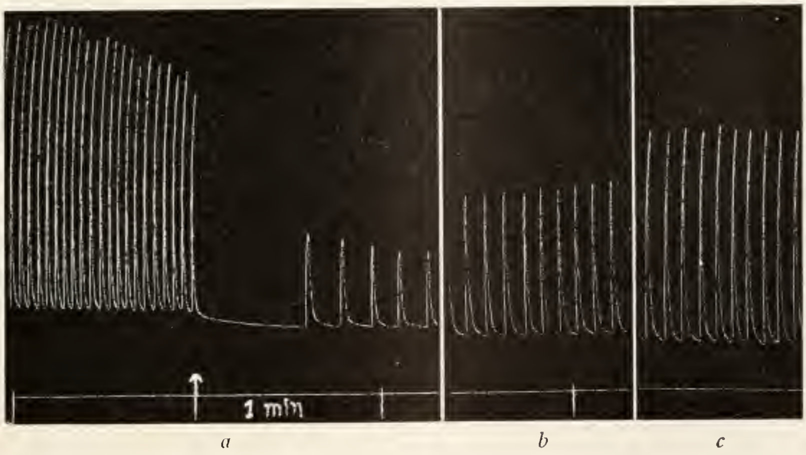

FIG. 6. Experiment No. 32. (*a*) Thermo-paradox obtained on change from a warm Ringer's solution at 41.5° C. to another Ringer's solution at room temperature (24.0° C.). (*b*) Seven minutes later; and (*c*) 14 minutes later at the room temperature.

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[7] The changes obtained on transfer from a pure NaCl solution to a normal Ringer's solution resemble the K-paradox rather than the Ca-paradox, for usually both the rate and the amplitude undergo the primary and temporary decrease.

creased. On bringing it back to the room temperature, the rate would be expected to return either rapidly, or gradually, to normal. What actually happens, however, is of the general nature designated as paradoxical—both the rate and the amplitude are immediately decreased to a level below normal, or the heart-beat may be completely arrested for a short period; then the normal rhythm for the room temperature is gradually resumed. This primary and temporary inhibition of the cardiac rhythm on change from a high to a low temperature is called the thermo-paradox. In general this effect is more pronounced the greater the decrease in temperature. When the temperature was suddenly decreased from 41.5° to 24.0° (Fig. 6), the heart-beat was immediately arrested for about half a minute and a gradual recovery followed. When the temperature was then decreased from 33.5° to 24.0° on the same ganglion, the heart-beat was greatly decreased both in rate and in amplitude, but not completely arrested, and the recovery was complete in a relatively short period.

Discussion

The demonstration of K-paradox, Ca-paradox and thermo-paradox in the ganglion of the *Limulus* heart as well as in the vertebrate heart offers additional evidence of the general physiological similarity between these hearts. The paradoxes observed in the ganglion of the *Limulus* heart differ, however, from those of the vertebrate heart in that while in the former both rate and amplitude may be involved in the primary temporary inhibition, in the latter the primary inhibition affects the rate only. This difference is to be expected when one considers that the vertebrate heart is strictly of the all-or-none type; while in the *Limulus* heart the amplitude of the contractions may vary greatly, apparently in correlation with the number of nerve cells acting simultaneously in the ganglion. A decrease in amplitude means simply a decrease in the number of active nerve cells. With the exception of this apparent difference and a few minor points, the paradoxes found in the ganglion of the *Limulus* heart are essentially identical with those described for the vertebrate heart.

Many explanations have been suggested for the paradox phenomena. In connection with his work on physiological radioactivity Zwaardemaker (1917, 1921) attempted to explain his radio-physiologic paradoxes in terms of the antagonistic actions between the light and the heavy radioactive substances when applied successively. Libbrecht believed that the K-paradox is a specific K-effect connected with the ability of the tissue to adapt itself to changes in environmental conditions. In his paper on the rabbit's heart, Busquet pointed out certain resemblances between the K-paradox and the effects of vagus stimulation. Yet the K-paradox can still be produced after the action of the vagus nerve is abolished by atropine (Busquet, 1921, and Kisch, 1927). Witanowski (1926) regarded a change in the permeability of the cell membrane to K-ion in the K-free solution as the essential factor in the production of the K-paradox. But the Ca-paradox, Sr-paradox, and thermo-paradox cannot be satisfactorily explained on these theories; and the demonstration of these paradoxes in the ganglion of the *Limulus* heart and in other tissues with automatic rhythmic activity indicates that the phenomenon is a general one, and that some general, or unitary mechanism is involved. Two distinct and equally important factors appear to be concerned: (1) a preliminary process in a modified medium, and (2) a process connected with the sudden return to the normal medium. The paradox appears almost simultaneously with the change of the solutions, indicating apparently a surface phenomenon. The preliminary process (*e.g.*, the immersion in a K-free, or a Ca-free, or a warm Ringer's solution) is preparatory in nature and requires a minimum duration; its effect is to establish a new equilibrium in the tissue such that on subsequent return to its normal medium a primary and a purely temporary inhibition of activity occurs before the tissue can resume its normal condition, *i.e.*, during the temporary period of disequilibration in the second solution. This is the essence of the paradox phenomenon.

SUMMARY

Potassium-paradox, calcium-paradox, and thermo-paradox have been observed in the ganglion of the *Limulus* heart.

The Ca-paradox is obtained on change from a Ca-free to a normal Ringer's solution, and the thermo-paradox is obtained on change from a warm to a cold Ringer's solution.

The K-paradox is obtained in a similar way on change from a K-free to a normal Ringer's solution, and its production depends upon the following factors:

1. The K-paradox is more readily obtained on the second or third repetition than on the first immersion of a fresh ganglion in the K-free Ringer's solution.
2. The paradoxical effect becomes more pronounced with increasing duration of the preliminary immersion in the K-free Ringer's solution.
3. The factor determining the K-paradox is the sudden change in the K-content of the medium surrounding the ganglion, and this change must be in the direction of increasing the K-content in the second solution.

4. The presence of a minimum amount of NaCl in the K-free Ringer's solution (about 30 per cent of the normal concentration) is necessary for the production of the K-paradox. The presence of $CaCl_2$ in the K-free, or the K-containing, solution is of only secondary importance perhaps by virtue of its antagonistic action.

In general the paradoxes found in the ganglion of the *Limulus* heart are essentially identical with those described in the vertebrate heart.

The author wishes to express his profound gratitude to Dr. R. S. Lillie for invaluable advice and much help.

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HEMATOLOGICAL STUDIES IN DOGFISH (MUSTELUS CANIS) [1]

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(From the Marine Biological Laboratory, Woods Hole, Mass., and the New York Hospital and the Department of Medicine, Cornell University Medical College)

I. The Normal Blood Picture and the Effect of Removal of Blood and of Turpentine Injections

In the mammal the blood-forming organs, and therefore the blood-cell picture, respond rapidly to environmental changes. This is seen in infections (Goodner, 1928; Reznikoff, 1929), severe exercise (Garrey and Butler, 1929) and in many conditions involving nutritional disturbances (Keefer, 1932). Certain blood cells, for example the neutrophiles, vary in number and in degree of maturation markedly and very soon after the onset of hemorrhage or the inoculation of bacteria. Since fish lack those white blood cells which form the important class of polymorphonuclear leucocytes in the mammal, it was thought of interest to determine what cells take their place, if the same agents were used which provoke an outpouring of these cells in mammals. Incidentally, some standard hematological values might be obtained by using the ordinary quantitative clinical pathological methods of enumeration and estimation.

Dogfish (*Mustelus canis*) were selected for this study because of their availability and because they could be manipulated outside the water for a considerable time without any appreciable struggle involving asphyxia. Blood was removed from the heart in small amounts, 0.5 to 0.75 cc., with a syringe and needle which had previously been rinsed with 1 per cent aqueous heparin solution. This prevented clotting effectively without diluting the blood appreciably. Total red and white cell counts were made using Toison's solution as a diluent with a standardized pipette and in a Hellige improved Neubauer hemocytometer. Hemoglobin readings were made with a Sahli hemoglobinometer standardized to give 14.5 grams of hemoglobin per 100 cc. as 100 per cent. Differential counts were made by the supravital method following the technique of Sabin (1929).

In this study six fish were used for post-mortem study, no blood be-

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[1] We wish to express our thanks to Dr. N. C. Foot and to Miss A. M. McDowell for their help in the pathological work of this study.

ing removed prior to their death. Twelve fish were used to determine the effect of frequent removal of blood from the heart upon the blood picture. Four fish were injected intraperitoneally with 1.5 cc. of sterile distilled water. Eight fish were injected intraperitoneally and one intramuscularly with turpentine in doses varying from 0.25 to 1.5 cc. Most of the injections were in 1 cc. amounts.

The initial counts in twenty-five fish gave the results shown in Table I.

TABLE I

Blood-counts in Twenty-five Normal Dogfish

	Average	Range
Hemoglobin	30 per cent (4.35 grams per 100 cc. of blood)	19–41 per cent
Red blood cells	393,000 per cu. mm.	210,000–630,000
White blood cells	97,000 per cu. mm.	60,000–178,000
Differential:		
Small lymphocytes	54 per cent	35–71 per cent
Large lymphocytes	9 " "	2–17 " "
Thrombocytes	18 " "	6–32 " "
Eosinophiles	14 " "	5–32 " "
Pseudoeosinophiles	4 " "	1–8 " "
Monocytes	1 " "	0–2 " "

Removal of blood from the heart infrequently or in small amounts caused no change in the red cell count and hemoglobin percentage. In most cases, however, some reduction in the white blood cell count was obtained, usually slight to moderate. Practically no variation occurred in the differential count except that when blood was withdrawn shortly before a fish died, the thrombocytes increased in number and the eosinophiles decreased. This, however, is found as a rule in all fish before death regardless of the cause. An illustration of the results obtained in a case where only small amounts of blood were removed is shown in Table II.

On the other hand, much loss of blood results in a marked decrease in the red cell count and in the hemoglobin percentage. The white cells are markedly depressed without any striking change in the differential count. Table III illustrates such a case. From this fish much blood was taken for the first count and considerable oozing of blood occurred after the puncture.

Injection of 1.5 cc. of sterile distilled water intraperitoneally has apparently no effect upon the blood picture of the fish. They showed no changes other than those seen when blood is removed for successive counts or which occur just before death in any fish.

TABLE II

The effect on the blood picture of removing small quantities of blood (less than 0.75 cc.). Fish No. 28.

Time of removal of blood	Hemo-globin	R.B.C.	W.B.C.	Differential count					
				Lympho-cytes	Large Lymph.	Thrombo-cytes	Eosino-philes	Pseudo-eosin.	Mono-cytes
	per cent	*per cu. mm.*	*per cu. mm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
8/16/33 9:00 A.M.	30	364,000	80,000	53	11	17	17	2	0
8/17/33 9:00 A.M.	32	349,000	65,000	39	16	24	18	2	1
8/18/33 9:00 A.M.	33	392,000	68,000	52	7	26	14	1	0
8/19/33 9:00 A.M.	30	393,000	76,000	55	3	30	10	2	0

TABLE III

The effect on the blood picture of removing large quantities of blood (more than 0.75 cc.). Fish No. 29.

Time of removal of blood	Hemoglobin	R.B.C.	W.B.C.	Differential count					
				Lymphocytes	Large lymph.	Thrombocytes	Eosinophiles	Pseudo-eosin.	Monocytes
	per cent	*per cu. mm.*	*per cu. mm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
8/16/33 9:30 A.M.	30	344,000	104,000	64	6	16	11	2	1
8/17/33 9:30 A.M.	$\boxed{18}$*	$\boxed{190{,}000}$	$\boxed{19{,}000}$	50	14	26	10	0	0
8/18/33 9:30 A.M.	24	272,000	52,000	54	10	21	13	2	0

* In Tables III and IV, heavy lines are drawn around numbers which show the striking variations due to the experimental procedure.

TABLE IV

The effect on the blood picture of injecting turpentine intraperitoneally

Time of removal of blood	Hemoglobin	R.B.C.	W.B.C.	Differential count					
				Lymphocytes	Large lymph.	Thrombocytes	Eosinophiles	Pseudoeosin.	Monocytes
	per cent	*per cu. mm.*	*per cu. mm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Fish No. 12									
8/8/13 4 P.M.	35	559,000	101,000	50	5	12	27	6	0
0.5 cc. turpentine intraperitoneally 4:45 P.M.									
7:30 P.M.	39	425,000	61,000	45	9	22	23	1	0
8/9/33 9 A.M.	$\boxed{19}$	$\boxed{344,000}$	$\boxed{28,000}$	$\boxed{78}$	3	$\boxed{6}$	$\boxed{8}$	3	2
2 P.M.	17	252,000	30,000	70	26	1	3	0	0
Dead 2:45 P.M.									
Fish No. 13									
8/8/33 8 P.M.	30	217,000	63,000	37	4	27	23	8	1
1 cc. turpentine injected intraperitoneally 9:45 P.M.									
8/9/33 9:10 P.M.	32	416,000	70,000	65	5	24	$\boxed{3}$	2	1
6:45 P.M.	20	207,000	$\boxed{25,000}$	$\boxed{79}$	6	$\boxed{6}$	$\boxed{5}$	3	1
8/10/33 9 A.M.	33	321,000	47,000	67	5	$\boxed{24}$	2	1	1
Dead 6 P.M.									
Fish No. 15									
8/10/33 11:30 A.M.	21	327,000	78,000	55	10	17	9	8	1
1 cc. turpentine intraperitoneally 12:20 P.M.									
8:20 P.M.	19	142,000	$\boxed{23,000}$	66	13	$\boxed{1}$	14	4	2
8/11/33 9 A.M.	19	320,000	73,000	54	14	25	$\boxed{3}$	3	1
6:45 P.M.	20	237,000	26,000	$\boxed{80}$	13	$\boxed{4}$	$\boxed{1}$	1	1
Dead 7:30 P.M.									

TABLE IV—*Continued*

Time of removal of blood	Hemoglobin	R.B.C.	W.B.C.	Differential count					
				Lymphocytes	Large lymph.	Thrombocytes	Eosinophiles	Pseudoeosin.	Monocytes
	per cent	*per cu. mm.*	*per cu. mm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Fish No. 16									
8/10/33 8 P.M.	36	359,000	127,000	37	9	20	32	1	1
1 cc. turpentine intraperitoneally 8:50 P.M.									
8/11/33 9:15 A.M.	32	393,000	$\boxed{70,000}$	$\boxed{79}$	3	$\boxed{13}$	$\boxed{4}$	1	0
7:30 P.M.	23	308,000	71,000	71	9	12	5	3	0
8/12/33 9 A.M.	33	432,000	75,000	80	4	11	4	1	0
Dead 12 M.									
Fish No. 18									
8/14,33 9 A.M.	32	442,000	178,000	69	8	6	15	1	1
0.25 cc. turpentine intraperitoneally 10:45 A.M.									
8/15/33 9 A.M.	23	319,000	$\boxed{38,000}$	70	2	$\boxed{21}$	$\boxed{4}$	3	0
Dead 8/16/33 A.M.									
Fish No. 19									
8/14/33 9:10 A.M.	41	423,000	146,000	51	15	19	13	2	0
0.25 cc. turpentine intraperitoneally 10:50 P.M.									
8/15/33 9:10 A.M.	36	333,000	$\boxed{87,000}$	$\boxed{66}$	8	20	$\boxed{3}$	2	1
Dead 8/16/33 A.M.									

When turpentine is injected intraperitoneally in fish in amounts mentioned previously, very definite changes occur which cannot be explained on the basis of the control studies (removal of blood, injection of distilled water). Invariably a distinct leukopenia ensues and the differential picture shows a marked increase in the percentage of lymphocytes, an immediate and persistent decrease of eosinophiles and usually an initial decrease of thrombocytes which in some fish may increase just before death and in others remain low. The difference between this picture and the usual ante-mortem differential count is that many of the fish which received injections of turpentine showed blood changes long before they died and while they were in excellent condition. This healthy state can be judged by activity and the color of their skin, which shortly before death, or if the fish is in poor condition, loses its dark color due to contraction of the chromatophores.

Table IV shows the results obtained with some of the fish which were injected with turpentine intraperitoneally.

TABLE V

Post-mortem Differential Counts on Fish

	Lymphocytes	Large lymphocytes	Thrombocytes	Eosinophiles	Pseudo-eosinophiles	Monocytes
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Fish No. 28	75	1	18	1	1	0
Fish No. 31	77	4	17	1	0	1

The results of examination of the intraperitoneal exudate before as well as after death showed a tremendous outpouring of lymphocytes, small, intermediate, and large, many of them full of phagocytized granules, globules, probably of turpentine, and bacteria. In some cases the exudate had the appearance of actual pus. One of the surprising findings was that bacteria and exudate were present in fish which died "naturally," that is, from which no blood was withdrawn and in which no needle had been inserted into the peritoneal cavity before death. In one control fish which jumped out of the tank and was found dead the next morning, no exudate and bacteria were found in the peritoneum.

Some of the fish showed bacteria in the blood just before death and in the post-mortem blood.

In two of the fish post-mortem differential blood smears showed a picture which is illustrated by Table V.

The high lymphocyte count, the moderate number of thrombocytes, and the almost complete repression of eosinophiles in the post-mortem blood emphasizes further the trend toward such a blood picture as death approaches.

It is, of course, important to see if the change in the blood picture due to impending death or due to the injection of a substance like turpentine was a reflection of the condition of the spleen, the hematopoietic organ of the fish. Supravital smears from freshly cut surfaces of the spleens of these fish immediately after death showed in the fish injected with turpentine an almost complete absence of eosinophiles. Smears from the spleens of normal fish showed a considerable number of eosinophiles. Fixed tissue studies demonstrated that in the injected fish, compared to controls, the spleens either had a marked diminution of eosinophiles or that these cells were not compact and the eosinophilic granules were swollen and took a faint dull pink stain. There was no question but that there was a distinct hyperplasia of the lymphocytic elements which showed hyperchromatic characters in the injected fish.

Discussion

Many features of these studies merit discussion. It is significant, for example, to note that the rather high eosinophile count in these dogfish under normal conditions may be associated with the prevalence of parasitic infestation (Stunkard, 1933).

In comparing the blood picture of the dogfish with that of the mammal under similar conditions of treatment, it is evident that the lymphocyte takes the place of the mammalian neutrophilic granulocyte or polymorphonuclear cell as far as its response to injury, its phagocytic ability, and its ameboid properties are concerned. Moreover, it is apparent that the so-called granulocyte is really a large lymphocyte, as suggested by Jordan and Speidel (1924), since all transition stages can be traced between the two. Perhaps the most interesting phase of this study is the persistent leukopenia seen also at the beginning of infections in mammals and sometimes extending throughout the course of disease. In the dogfish apparently this leukopenic stage does not pass over into leukocytosis. The increased production of lymphocytes as evidenced by hyperplasia of the spleen results in a concentration of these cells at the site of irritation,—in this study, the peritoneal cavity. In mammals Ewing (1895) and Doan and his coworkers (1928) found a marked increase of cells in the spleen and liver in the early stages of infection. But as the disease progressed the blood reflected the increase of cell production in most cases by showing a marked increase in cells. It would be of interest to determine where in the phylogenetic scale this ability to change from a leukopenia to a leukocytosis occurs. In a disease known as acute glandular fever in humans an excessive response of lymphocytes, as in these fish, is also seen. These problems may have considerable interest to the comparative pathologist.

CONCLUSIONS

1. The blood picture in twenty-five dogfish (*Mustelus canis*) has been studied and the findings recorded.

2. Frequent removal of blood or removal of blood in large amounts from fish may cause an anemia and a leukopenia but produces no change in the relative numbers of the different white blood cells.

3. Injection of sterile distilled water intraperitoneally causes no change in the blood picture.

4. Injection of turpentine intraperitoneally causes a distinct leukopenia, a relative increase of lymphocytes, usually a decrease of thrombocytes and always a marked decrease of eosinophiles.

5. Just before death control fish, as well as those which had been injected with turpentine, may show an increase of thrombocytes.

6. Studies of the peritoneal exudate and the spleen, as well as of the blood, indicate that turpentine acts as a definite chemotactic agent stimulating production of lymphocytes. The lymphocyte is the actively responding cell to acute irritation in the dogfish and has the same significance as the neutrophile (polymorphonuclear) in mammals.

7. Antemortem and postmortem studies suggest that bacterial invasion of the blood stream and of the peritoneal cavity is not uncommon even in control fish and that blood changes (increase of lymphocytes and decrease of eosinophiles) may occur just before death.

8. This investigation indicates that the changes observed may be of importance in comparative biological problems.

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FURTHER OBSERVATIONS ON THE SALT REQUIREMENTS OF LIGIA IN BERMUDA[1]

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A previous paper (Barnes, 1932) dealt with the salt requirements and space orientation of the littoral isopod *Ligia baudiniana* in Bermuda. It was shown that *Ligia* is unable to withstand prolonged immersion in sea water and survives best in moist air. The isopod is poikilosmotic and dies within a few hours in distilled water, fresh water, and sea water of half dilution or less. Its survival in natural sea water is not much longer than in artificial sea water even if the latter lacks Mg. Experiments with 5/8 M solutions of single salts established the toxicity series K $>$ Mg $>$Ca $>$ Na.

The present paper describes the longevity of *Ligia* in weaker solutions of single salts, in antagonistic solutions, and when living in air with access to salt solutions.

Habits

Although *Ligia* occurs in great numbers on most of the rocky shores of the islands, we have very rarely observed the animal in the act of feeding. Nicholls (1930–31) describes the nocturnal "browsing" of *L. oceanica,* which clings to *Fucus* and cuts off very small portions with its mandibles. During the summer of 1933 it was found that the feeding of *L. baudiniana* can be easily watched if the observer remains motionless on the rocks when the tide begins to recede. The isopods leave their hiding places and follow the ebbing tide, feeding on the algal covering of the damp rocks. Unlike *L. oceanica, L. baudiniana* appears during the day and is positively phototropic in the laboratory. After the intertidal zone is dried by the sun, they usually retreat under stones and in crevices but some remain near the water's edge. The animal scrapes off the unicellular algæ by a vertical motion of the head, the whole anterior half of the body taking part in the rasping movement. The animal feeds for a short interval and moves on for about 10 cm. and feeds again; this intermittent process is especially evident when the intertidal zone is first invaded.

In the preceding paper it was shown that *Ligia* must keep its gills moist for aerial respiration. Water will rise by capillarity up the spines and uropods or the gills may be brought into contact with a moist substratum by lowering the abdomen. This reflex is elicited by contact with sea water on any part of the body. If a drop of sea water is placed on the head of an animal which has been out of water some time, the uropods are first brought together and then lowered to the substratum, even if the latter is dry. This reflex is so strong that a running animal

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[1] Contribution from the Bermuda Biological Station for Research.

TABLE I

Longevity of Ligia in Air and in Water

Medium	Year	Average longevity	Maximum longevity	No. specimens tested
		hours	*hours*	
Distilled water	1932	4	5	21
	1933	3.9	5	33
	Both years	4	5	54
Fresh water	1932	7½	8	21
	1933	7	9	9
	Both years	7	9	30
Sea water	1932	34	297	93
	1933	74	240	25
	Both years	42½	297	118
2.5 cc. sea water plus 100 cc. H_2O	1933	4½	6	10
25% sea water	1932	6	10	6
	1933	6½	7½	24
	Both years	6½	10	30
50% sea water	1932	20	70	7
	1933	17½	64	15
	Both years	18	70	22
Air (dry bowls)	1932	11	12	8
	1933	17¾	25	49
	Both years	15¼	25	57
Air over damp sand	1932	360	625	31
	1933	384	648	51
	Both years	375	648	82

drags its spines when traversing a wet region. It is probable that *Ligia baudiniana* is kept moist by its habit of hiding in crevices or under fragments of the porous Bermuda rock which is saturated at high tide. Many specimens are so moist that they glisten in the sun, but I have never found submerged specimens. A pursued isopod will enter the water only if no other path of escape is available but soon returns to air. It is interesting to observe the mass migrations to upper regions of the shore as the tide advances. The individuals at the water's edge are the first to start landwards, but these are joined by specimens several feet from the water line. Individuals of all sizes exhibit this periodic retreat, the mature ones being in advance and always ascending the furthest inland. In captivity the young are released only by females kept in bowls of sea water, but the young (usually about eighty from one specimen) immediately crawl out of the water if any available projection is present. During the past summer the isopods were collected from the intertidal zone at low tide by shaking loose stones over a pail with smooth vertical sides. The specimens dealt with in the previous paper were taken on a wall several feet from the sea. This fact may help to explain differences in the longevity data for the two series (see Table I).

The rôle of positive geotropism as an important factor in the orientation of *Ligia* towards the sea when released some distance inland was confirmed and the tendency of half-grown specimens to crawl down slopes was again observed. In one experiment the isopods crawled toward the sea *up* a slight incline (sand) of 10°, but on a 30° slope in the same region they crawled down *away* from the sea. The orientation of the isopod to shore when released in the sea was studied in tidal pools. Isopods released in the center of a pool swim or crawl to the water's edge in all radial directions regardless of the direction of the mainland. In the water their slow swimming movements make them an easy prey to shore fishes but their rapidity on land enables them to escape pursuit by the mangrove crab *Goniopsis cruentatus*. However, *Ligia* does not occur in mangrove swamps where *Goniopsis* is very abundant.

Longevity of Ligia in Air and in Water

The number of individuals tested in some of the 1932 experiments was not large and it was necessary to repeat some of the tests, especially since the specimens were taken from a habitat nearer the water line. The same average longevity was found in distilled water,—4 hours,—and in fresh water, 7 hours, but the 1933 isopods lived longer in sea water (Table I). However, we regard the average of the two series, 42 hours, as a satisfactory working average for comparison with other media. The 1933 series of isopods in dry dishes lived an average of 17¾ hours, 6¾ hours longer than the 1932 series. This was probably due to the fact that the 1932 isopods were collected several feet above the high water line and had been exposed to desiccation. In air over damp sand the new series gave a slightly greater longevity, 384 as compared to 360 hours. Moulting was observed only in these specimens and not in submerged ones. The duration of life in 25 per cent and 50 per cent sea water, 6 and 18 hours respectively, was nearly the same in each series. In general it will be seen that the same relative effect was secured in the various media in spite of the difference in year, number of specimens, habitat, and method of collecting. In both series individual specimens were tested in bowls containing 100 cc. of solution.

TOXICITY OF SINGLE SALTS

The survival of *Ligia* was determined in distilled water containing the percentage of a single salt occurring in sea water for comparison with the toxicity series of 5/8 M single salts previously described. In a solution containing 2.5 cc. of 5/8 M NaCl plus 100 cc. of water the average duration of life was only 2¾ hours with a maximum of 3½ hours, while in $CaCl_2$ of the same concentration the average longevity was 9¼ hours with a maximum of 11 hours (Table II). The results are of interest since NaCl is the least toxic salt in solutions isosmotic with Bermuda sea water. A 5/8 M NaCl series was repeated and the same average longevity, 8 hours, obtained as previously, showing that the 1933 specimens did not differ in their reaction to sodium chloride. In 2.2 cc. of 5/8 M KCl plus 100 cc. of water, *Ligia* lived an average of 3 hours with a maximum of 4 hours, and durations of approximately the same order were found for 50 cc. 5/8 M KCl plus 100 cc. water, *i.e.*,

TABLE II

Longevity of Ligia Immersed in Solutions of Single Salts

Medium	Average longevity	Maximum longevity	No. specimens
	hours	*hours*	
5/8 M NaCl 1933	8	9	18
5/8 M NaCl 1932	8	14	12
Both years	8	14	30
2.5 cc. 5/8 M NaCl plus 100 cc. H_2O	2¾	3½	10
2.5 cc. 5/8 M $CaCl_2$ plus 100 cc. H_2O	9¼	11	30
2.2 cc. 5/8 M KCl plus 100 cc. H_2O	3	4	27
50 cc. 5/8 M KCl plus 100 cc. H_2O	2¼	2½	10
11.6 cc. 5/8 M $MgCl_2$ plus 100 cc. H_2O	7	9	13

TABLE III

Longevity of Ligia in Antagonistic Solutions

Solution	Average longevity	Maximum longevity	No. of specimens tested
	hours	*hours*	
Artificial sea water (1932)	40	123	8
5/8 M artificial sea water lacking NaCl (1932)	2	2	5
Same (1933)	2¾	3½	12
Artificial sea water lacking NaCl (NaCl replaced by distilled water)	5¾	9	20
5/8 M artificial sea water lacking NaCl and $MgCl_2$	1¾	2½	10
Artificial sea water lacking Na and Mg (NaCl and $MgCl_2$ replaced by distilled water)	9½	12	17
1 part 5/8 M NaCl plus 1 part 5/8 M $CaCl_2$	24¾	28	4
4 parts 5/8 M NaCl plus 1 part 5/8 M $CaCl_2$	51½	96	15
2.5 cc. 5/8 M $CaCl_2$ plus 100 cc. 5/8 M NaCl	21	40	20
1 part 5/8 M $CaCl_2$ plus 1 part 5/8 M KCl	4	6	10
2.2 cc. 5/8 M KCl plus 100 cc. 5/8 M NaCl	10½	14	10
4.4 cc. 5/8 M KCl plus 100 cc. 5/8 M NaCl	6½	9	10
11.6 cc. 5/8 M $MgCl_2$ plus 100 cc. 5/8 M NaCl	9¼	18	20

average 2¼ hours, maximum 2½ hours. It was reported in the first paper that *Ligia* lives an average of 1½ hours in 5/8 M KCl in which the gills are completely paralysed. In the weak KCl solution, however. the gills vibrated normally but were stopped in the solution containing 50 cc. of 5/8 M KCl plus 100 cc. of water. In a solution containing the percentage of Mg in sea water (11.6 cc. of 5/8 M $MgCl_2$ plus 100 cc. of distilled water), the average longevity was 7 hours and the maximum 9 hours. It is clear that the toxicity series in weak solutions is quite different from that in solutions isosmotic with Bermuda sea water.

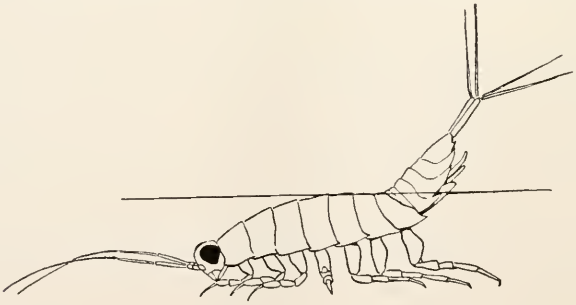

FIG. 1. A specimen in a solution of equal parts NaCl and KCl 5/8 M in which the gill beat is inhibited by the K. The animal has elevated its gills out of the water for aerial respiration.

IONIC ANTAGONISM

In the first paper it was mentioned that no satisfactory binary mixture was found but antagonism seemed evident between Na and Ca. Further work with salt mixtures supports this statement (*vide* Table III). The most favorable of the binary solutions tested was composed of 80 per cent NaCl and 20 per cent $CaCl_2$ in 5/8 M concentration.

It was of interest to observe that the gills vibrated in the weak KCl solutions, thus showing the toxicity of the potassium ion apart from its inhibition of the gill beat, which is completely inhibited in 5/8 M KCl made in sea water, 5/16 M KCl, and in equal parts of 5/8 M KCl and NaCl. On the other hand, the gill rhythm occurs in the following solutions: 2.2 cc. 5/8 M KCl plus 100 cc. H_2O; 2.2 cc. 5/8 M KCl plus 100 cc. 5/8 M NaCl; 4.4 cc. 5/8 M KCl plus 100 cc. 5/8 M NaCl; and in M/4 KCl made in sea water. In solutions inhibiting the gill beat the isopod sometimes elevates its moist gills out of the water for aerial respiration. (Fig. 1.)

TABLE IV

Longevity of Ligia in Dry Bowls Containing Watchglasses of Various Solutions

Solution in watchglass	Average longevity	Maximum longevity	No. specimens tested
	hours	*hours*	
Sea water	292¾	432	13
Distilled water	156	240	19
25% sea water	240	432	8
1 part 5/8 M NaCl plus 1 part 5/8 M KCl	10	19	14
5/8 M NaCl	60	144	16
5/8 M $CaCl_2$	29	52	22
2.5 cc. 5/8 M $CaCl_2$ plus 100 cc. H_2O	156	216	18
5/8 M KCl	8½	24	24
2.2 cc. 5/8 M KCl plus 100 cc. H_2O	168	216	10

LONGEVITY OF LIGIA HAVING ACCESS TO SALT SOLUTIONS

Watchglasses containing sea water or other solutions were placed in dry bowls containing an isopod (Table IV). In the less toxic solutions the isopod remained at the edge of the watchglass. This was observed with sea water, 5/8 M NaCl, 5/8 M $CaCl_2$, 2.5 cc. of 5/8 M $CaCl_2$ plus 100 cc. of water and 2.2 cc. of 5/8 M KCl plus 100 cc. of water. When the watchglass contained sea water, the isopod crawled through the solution so often that after a few days most of the sea water was in the bowl. In such cases the animal remained in the watchglass if this contained less water than the bowl.

As will be seen in Table IV, the animals lived for long periods with access to sea water, even when diluted to 25 per cent, and to distilled water. It was again possible to demonstrate the toxicity of KCl apart from its paralyzing action on the beating of the pleopods in submerged specimens.

Discussion

It is of interest to compare the survival of *Ligia baudiniana* in hypotonic solutions with that of *L. oceanica* which appears to be somewhat less terrestrial since it sometimes remains covered by the tide. According to Tait (1916–17) and Nicholls (1930–31), *L. oceanica* survives for very long periods when kept in sea water. Its longevity in diluted sea water is also greater than that of *L. baudiniana,* Tait (1916–17) reporting a survival of as long as forty days in 50 per cent sea water and ten days in 25 per cent sea water (compare with Table I). However, both forms soon succumb in distilled water, the maximum being 5 hours for *L. baudiniana* and 36 for *L. oceanica.* Bateman (1933) finds that the blood of *L. oceanica* is approximately equal in concentration to sea water and that the isopod shows high osmotic independence for a short time when immersed in hypotonic sea water. Animals living on moist sea weed had a salt concentration of 0.631 M, rather greater than sea water. Nicholls (1930–31) kept *L. oceanic*a for more than 15 months in moist air with *Fucus.* He reports a more rapid flapping of the pleopods in hypotonic sea water. In one instance 2 liters of sea water containing an isopod were diluted by 25 cc. each day. The time for ten beats decreased from 6 seconds on the fourteenth day to 5 seconds in a month's time. The frequency of gill rhythm of *L. baudiniana* also increases in diluted sea water. The time for ten beats decreased from 3 to 2.7 seconds in a sample specimen when transferred to 50 per cent sea water. Bateman (1933) found that the osmotic pressure of the blood in *oceanica* remained almost normal for several hours in this dilution but after 19 hours the osmoregulation broke down. The increased gill frequency can be interpreted in terms of Schlieper's (1929) theory, as a means of providing the extra oxygen necessary for work performed in the temporary resistance to osmotic forces. The investigations of Fox and Simmonds (1933), for example, show a higher oxygen consumption in species of *Gammarus* inhabiting fresh water.

According to Bogucki (1932), the marine isopod *Mesidotea entomon* exhibits considerable osmoregulation in hypotonic sea water and survives a few days in fresh water and 20 hours in distilled water. It contrasts rather sharply with *Ligia baudiniana,* which has less in common with fresh water animals.

The toxicity series for single salts isosmotic with Bermuda sea water is appreciably different from that for hypotonic solutions of the chief cations. The 5/8 M toxicity series K > Mg > Ca > Na becomes, in the weak solutions, Na > K > Mg > Ca. In the hypotonic solutions permeability is an important factor governing the loss of salts from the blood and Na, the least toxic ion in isosmotic concentration, becomes the most toxic, probably in its action of increasing permeability. In the same way Ca prevents the leaching action of water as Pantin (1931) has described in the estuarine worm *Gunda*. A similar effect has been described by McCutcheon and Lucké (1928) on sea urchin eggs and indeed was shown years ago by Osterhout (1915) in plant cells. *Ligia baudiniana* lives twice as long in 2.5 cc. of 5/8 M Ca plus 100 cc. water as in 2.5 per cent sea water (Tables I and II).

In binary mixtures with Na in natural proportions the Ca solution is the most favorable, but it is interesting to note a greater longevity when the Ca is increased to 20 per cent. Ca, however, does not antagonize the toxicity of K to a great extent (Table III) and in Na-K mixtures the longevity appears to be determined by the amount of K present.

The watchglass experiments showed the same toxicity series for solutions isosmotic with Bermuda sea water as in the immersion tests. With access to weak solutions, however, the isopods exhibited the same longevity with K and Ca. A mixture of equal parts of Na and K showed little antagonism in 5/8 M concentration, the average duration of life being one-thirtieth of that observed when the animals had access to sea water. The amphibious habits of *Ligia* render the isopod particularly suited for the study of specific ion effects which can be determined apart from osmotic and permeability disturbances.

SUMMARY

1. The toxicity series for the chief cations in sea water for *Ligia baudiniana* changes with the concentration of the single salts. In a weak solution Na is the most toxic but in 5/8 M concentrations it is the least toxic ion.
2. It is suggested that this is due to permeability effects in hypotonic solutions.
3. Na and Ca gave the most favorable binary mixture.
4. When kept in air with access to solutions of single salts *Ligia* shows the same toxicity series with respect to K, Na, and Ca as when immersed in isosmotic solutions.

I am indebted to Miss A. K. C. Barnes for valuable assistance with the experiments.

It is a pleasure to express my gratitude to Dr. J. F. G. Wheeler, who placed the facilities of the Bermuda Station at my disposal.

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THE CONCENTRATIONS OF PROTEASES, AMYLASE, AND LIPASE IN CERTAIN MARINE FISHES

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Marine fishes show great diversity as to food, feeding habits, special habitat, and rate and type of metabolism. Some have predominantly a fatty metabolism while others have essentially a carbohydrate metabolism in the sense that the nutritive reserves are stored in the form of fat or carbohydrates. Certain fishes, such as the mackerel, are extremely active; others are very sluggish; still others represent all the intergrades. Great variation is shown as to the development of the different organs concerned with digestion; in some fishes the pancreas is more or less clearly defined, in others it is diffuse and may be partially included in the liver. Pyloric cæca, which are the embryological equivalent of the exocrine pancreas, may also be its physiological equivalent in some cases. These structures are absent in many fishes.

As these factors vary, it is to be expected that there will be adaptive variations in the amounts and sites of production of the different digestive enzymes secreted. It is the purpose of this investigation to determine where the enzymes are secreted, and in what quantities, with a view to making correlations with the anatomy and physiology of the fishes studied.

The early literature bearing upon digestion in fishes is well reviewed by Yung (1899) and Sullivan (1907).

The rate of digestion has been studied by Van Slyke and White (1911). They state that in the dogfish two to three days are required for the disposal of a meal; this is about six times as long as the period in mammals, which may be due to the temperature difference. Svolima (1919) found the period of digestion to last up to five days. Yung (1899) had set the period at ten hours.

Kenyon (1925) compared digestion in the different classes of vertebrates. Peptic digestion he found to be remarkably uniform among all the animals investigated, with the exception of the stomachless carp. Gastric acidity is exceedingly variable, apparently depending upon the functional state of the organ. There seems to be no correlation between peptic activity and food habits. There is a close correlation between the structure of the tract and the distribution of the enzymes. Amylase and invertase are present in herbivorous fishes to a larger extent than in carnivorous. Apparently there has been little change in the character of the enzymes or their rates of activity in the evolution of the amphibians, reptiles, and mammals from primitive types.

The production of relatively large amounts of carbohydrases in the herbivorous carp, as contrasted with the carnivorous pike, has also been pointed out by Vonk (1928).

It is generally agreed that the enzymes of fishes are similar to those of warm-blooded animals. The subject is reviewed by Chesley (1934) in a paper dealing with the temperature coefficients of amylases of different origins. It was found that the optimal temperatures (obtained under comparable, rigidly controlled conditions) were somewhat lower for the amylases of cold-blooded animals. The order found for the optimal temperatures is human amylase > terrapin amylase > fish amylase. Also it was found that fish amylase is relatively much more effective at 3.5° C. than either of the other two; human amylase is intermediate.

This latter finding does not accord with Riddle's (1909) general conclusion that " higher forms " progressively lose the power of digestion at lower temperatures. Riddle studied peptic digestion in Mett's tubes introduced into the stomachs of living animals kept in constant temperature baths. He also concluded that there is a progressive loss in digestive power in successively higher forms.

MATERIAL

Fishes used in this study were selected as representatives of diverse specializations, as indicated in the introduction.

(1) Very active:
    The mackerel, *Scomber scombrus* Linnæus.
    The menhaden, *Brevoörtia tyrannus* (Latrobe).
    The scup, *Stenotomus chrysops* (Linnæus).
(2) Intermediately active:
    The sea robin, *Prionotus carolinus* (Linnæus).
    The puffer, *Spheroides maculatus* (Bloch and Schneider).
(3) Inactive or sluggish:
    The toadfish, *Opsanus tau* (Linnæus).

The mackerel, menhaden and scup represent active fishes, as shown by their rapid swimming, stream-line bodies, high blood sugars, high hemoglobin and blood iron concentrations and respiratory rates. The menhaden differs from the other two in storing much of its reserve as fat. The mackerel and menhaden feed by swimming rapidly near the surface, and straining their food out of the water. The mackerel is carnivorous, and exercises a degree of selection for *Calanus* (Bigelow and Welsh, 1924). The menhaden is predominantly herbivorous, feeding upon diatoms and peridineans (Bigelow and Welsh, 1924; Peck, 1894).

The toadfish is very sluggish; it lies at the bottom waiting for prey, and does little active hunting. The body is depressed; the blood sugar, hemoglobin and iron are all low. The respiratory rate is correspondingly low.

The sea robin and puffer are of intermediate activity as judged by these criteria (Hall, 1929; Hall and Gray, 1929; Gray and Hall, 1930). The sea robin stores a large amount of fat, and for this reason may be classified with the menhaden in type of metabolism.

Methods

Preparation of the Enzyme Solutions

The fishes used in this investigation were kept in live cars or tanks for a few days before using, in order to have them in approximately the same condition as regards gastro-intestinal contents. With the mackerel this scheme was impracticable, and fresh fish had to be used whenever available. The body-weights of all the fishes fell between 200 and 280 grams.

The viscera were separated, washed, slit open, washed again, blotted, and weighed. The tissues were ground vigorously with glass fragments. In many cases the isolation of the pancreas was a physical impossibility; here the procedure was simply to grind up the mesentery, after removing portions which obviously did not contain pancreatic tissue. Concordant values in enzyme quantities reckoned as units per gram of " pancreatic tissue " are hardly to be expected.

Bile was usually collected from the gall bladder by means of a needle and syringe. In many experiments, the gall bladder was stripped of its serosa and ground up in the bile, and the whole extracted.

The extracting agent was added to the tissue in the proportion of 20 ml. per gram of tissue. The whole was put into a small flask and allowed to stand at room temperature for twenty-four hours. Fifty per cent alcohol or 50 per cent glycerine were used exclusively after it had been established that these were the most efficacious of the solutions tried. The gastric mucosæ were extracted with N/10 HCl.

The several organs were separately and simultaneously extracted.

The Measurement of Digestion

Pepsin and Trypsin.—These proteases were estimated by the viscometric method described by Northrop and Hussey (1923). For pepsin, a 3 per cent gelatin preparation was used. The pH was maintained at 2.5. For trypsin, a similar gelatin solution was buffered at pH 7.4, using 0.05 M phosphate. The substrate (10 ml.) was put into an Ostwald viscosimeter in a water bath at 35° C.; 0.2 to 0.4 ml. of tissue extract was added when the gelatin had come to the temperature of the bath. Boiled enzyme was added to a similar system which served as a control. At intervals, the viscosities of the systems were determined. One unit of enzyme is defined as the amount which will cause a reduction of 20 per cent in the viscosity in sixty minutes. The reaction follows a unimolecular course and as a result one unit of enzyme causes a reduction of 10 per cent in 19 minutes, or 5 per cent in 8.2 minutes. Two units of the enzyme will cause a decrease of 20 per cent in viscosity in half the time required by one unit. An example of calculation will be given below.

Amylase.—For the determination of amylase, Davison's (1925) method was used. The limitations of the method have been discussed in an earlier paper (Chesley, 1931). The procedure was very similar to that used for the estimation of proteases. Sodium chloride was added to the system in a concentration of 0.05 M. The pH was kept at 7.2 by means of phosphate buffers. Units of enzyme are calculated in the same manner as for the proteases.

Lipase.—The Kanitz (1906) method was used. Ten milliliters of faintly alkalinized (NaOH) olive oil were incubated with 3 ml. of tissue extract for 16 hours at 40° C. To this were added, at the end of the digestion period, 50 ml. of acid-free alcohol and 5 ml. of ether, with three or four drops of phenolphthalein. The mixture was shaken and titrated with 0.1 N NaOH. A blank was run for each experiment. The amount of oleic acid liberated under the action of the enzyme was then calculated from the titration value.

EXPERIMENTAL RESULTS

Factors Influencing Enzyme Activity

Temperature.—The results of these experiments will be the subject of a separate paper. It was found that over a period of thirty minutes, under the conditions of pH and ionic concentrations which were maintained, the optimal temperature is 35° C. Accordingly, this temperature was kept throughout the whole work.

Hydrogen Ion Concentration.—Experiments were done only with amylase. The biliary amylase of several different fishes, acting at 35° C., was found to have a critical optimum at pH 7.2. The activities are considerably less at pH 7.0 and 7.4. This is shown in Table I; the values given are relative, the activity at pH 5.3 being set at unity. Sodium chloride was present in a concentration of 0.05 M, and phosphate buffers in an equal amount. These determinations were made viscometrically, and the optimum, which is atypically critical, may be governed in part by a pH effect upon the stability of the viscosity of the starch. Further investigation of pH effects upon fish amylase is planned.

As a routine procedure, the pH was maintained at 7.2 for all amylase determinations.

Sodium Chloride Concentration.—Since the chloride ion is the most efficacious of the anions activating amylase, and since the chloride concentration of the sea water is higher than the optimal activating concentration reported for mammalian amylase, the influence of chloride concentration upon fish amylase was studied, using three methods of measuring enzyme activity. The experiments were conducted as follows. Fifty-milliliter portions of 2 per cent soluble starch, buffered to pH 7.2 by means of 0.05 M phosphate mixture, were put into a series of flasks kept in the water bath at 35° C. Successively increasing quantities of a molar solution of NaCl were added to the preparations, and as soon as the temperature of the bath was attained, a definite quantity of enzyme solution was added. At intervals of a minute, five drops of the digest were removed and blown into 5 ml. of a N/8,000 iodine solution. The time was determined for the disappearance of the blue color reaction, and for the disappearance of any color reaction (referred to in Table II as "no blue" and "achromic" iodine methods respectively). At the end of 30 minutes, 5 ml. of the digest were removed and added to 95 ml. of Fehling's solution, and the maltose concentration was determined.

TABLE I

The effect of pH upon fish amylase activity. Temperature 35° C. Phosphate 0.05 M; NaCl 0.05 M. Time, 30 minutes. Determined viscometrically.

Source	Relative activity						
	pH : 5.3	5.9	6.6	7.0	7.2	7.4	7.7
Rudderfish	1	1.3	1.4	1.6	2.4	2.1	1.9
Toadfish	1	1.4	1.6	2.8	7.6	—	5.4
Puffer	1	1.7	2.2	2.7	3.4	3.2	—

The results, which are shown in Table II as relative activities at different chloride concentrations, indicate that the concentration of chloride which fully activates mammalian amylases (0.01 M, Sherman, Caldwell, and Adams, 1928) is not sufficient to activate fully fish amylase. In all routine determinations, the NaCl concentration was kept at 0.05 M, as this concentration fully activates the enzyme. Incidentally, these figures are not strictly quantitative, as the concentration of chloride impurities in the starch is unknown.

TABLE II

The effect of chloride concentration upon fish amylase activity. Temperature 35° C.; pH 7.2; phosphate 0.05 M. (Starch not chloride-free.)

Method	Relative activity					
	None	0.0025 M	0.0125 M	0.025 M	0.05 M	0.1 M NaCl
Iodine. No blue	1	1.70	2.38	2.70	2.77	2.80
Iodine. Achromic	1	1.82	2.20	2.50	2.67	2.70
Copper reduction	1	1.56	1.96	2.16	2.56	2.60
Mean	1	1.69	2.18	2.45	2.67	2.70

Concentration of Enzyme.—As is to be expected, fish amylase obeys the general rule (which does not apply to certain proteases) that the rate of hydrolysis is proportional to the concentration of enzyme. Twice as much enzyme effects the same change in half the time. This was determined viscometrically over a range between 0.02 and 0.6 ml. of tissue extract, and is shown in Table III. The definition of the enzyme unit is based upon this relationship.

TABLE III

The effect of enzyme concentration upon fish amylase activity. Temperature 35° C.; pH 7.2; phosphate 0.05 M; NaCl 0.05 M. Determined viscometrically.

Enzyme	Decrease in viscosity	Time required	Units per ml. enzyme sol.
ml.	*per cent*	*minutes*	
0.02	5	70	5.85
0.04	5	35	5.85
0.08	5	15	6.80
0.16	10	20	6.00
0.32	10	10	6.00
0.64	20	15	6.24

TABLE IV

Proteases. Units per gram of tissue. Temperature 35° C.; pH 2.3 and 7.4. Body weights 200 to 280 grams per fish.

Fish	Stomach	Intestine				Cæca		Pancreas		Liver		Bile and gall bladder		Total units in body (Approximate)
		Upper third		Second third		Units	Wt.	Units	Wt.	Units	Wt.	Units	Wt.	
		Units	Wt.	Units	Wt.									
Mackerel	—	287	0.96	—	—	19	4.25	587	0.63	13	3.47	—	—	760
Menhaden	Trace	40	0.41	Trace	0.38	142	3.54	140	0.37	Trace	0.73	23	1.16	580
Scup	16	228	0.62	Trace	—	238	0.18	150	0.31	68	0.23	46	2.34	340
Sea robin	24	44	1.14	—	0.95	98	1.00	103	0.54	Trace	3.18	77		250
Toadfish	22	Trace	—	Trace	—	—	0.00	2	0.45	28	7.60	Trace		210

These figures will serve to illustrate the method of calculation of units of enzyme. One unit of the enzyme, by definition, will cause a 5 per cent decrease in viscosity in 8.2 minutes. Since 70 minutes were required for 0.02 ml. of enzyme solution to produce a 5 per cent change in viscosity, there are present $8.2/70 = 0.117$ unit, and in one ml. of the enzyme solution there would be $0.117 \times 1/0.02 = 5.85$ units.

Digestion in the Fishes Studied

The Mackerel.—The mackerel is predominantly an animal plankton feeder, which means that it is an active fish. Its energy requirements would indicate that it should have a rapid and efficient digestion. That this is the case will be seen by reference to Tables IV, V, and VI, which show that the mackerel has the greatest amounts of enzymes. The pancreas, and secondarily, the intestine, are the chief sites of zymopoiesis. The pyloric cæca, of which there are about 190, produce very small quantities of enzymes except lipase. Amylase secretion is well localized in the pancreas.

TABLE V

Amylases. Units per gram. pH 7.2. Temperature 35° C. NaCl 0.05 M; phosphate 0.05 M.

Fish	Stomach	Intestine		Cæca	Pancreas	Liver	Bile	Total amylase
		Upper third	Second third					
Mackerel	Neg.	Trace	16	Trace	70	Neg.	Neg.	50
Menhaden	Neg.	12	24	21	60	Trace	6	40
Scup	Neg.	Neg.	—	Neg.	35	Trace	6	25
Sea robin	Neg.	Neg.	Trace	7	Trace	Trace	20	20
Puffer	Neg.	20	—	—	35	40	17	—
Toadfish	Neg.	Trace	3	—	Trace	Trace	5	Trace

The Menhaden.—The menhaden is another active fish, being a plankton feeder. As an adaptation to its plant diet, the menhaden intestine is much longer than that of any of the other fishes studied; also the stomach is remarkable for its heavy muscular walls and horny lining.

There is a large amount of body fat, to which the fish owes its economic importance. In view of their fatty metabolism, and the fats and oils in the diet, one might expect to find relatively much lipase in the digestive system. This is not the case.

In protease content, the menhaden is second only to the mackerel. The large number of cæca are important as a source of enzymes. The data are shown in Tables IV, V and VI.

The Scup.—A third fish of the active group was studied. Tables IV, V, and VI show that the scup has nearly as much protease, per gram of tissue, as the menhaden, while lipase and amylase are present in considerable amounts. The four pyloric cæca, small as they are, seem to be important in the secretion of protease. The bile, with which the intestinal content is invariably deeply stained, possesses a considerable amount of protease and some amylase. Apparently the bile is an important factor in the scup's digestion; the gall bladder is relatively large and always distended with enzyme-rich bile.

The Sea Robin.—This fish stores fat, and in the great majority of cases, the liver is cream-colored and greasy, indicating large amounts of liver fat. The gastro-intestinal lipase is slight in quantity, as in the menhaden; in fact it is the least found in any of the fishes studied (Table VI). The trypsin and amylase are correlated in quantity with the general activity of the fish (Tables IV and V).

TABLE VI

Lipases. Grams of oleic acid per gram of tissue. Alkaline olive oil. Temperature 40° C.

Fish	Stomach	Intestine		Cæca	Pancreas	Liver	Bile
		Upper third	Second third				
Mackerel	0.29	0.54	—	0.46	0.71	0.42	0.35*
Menhaden	—	0.14	0.12	0.12	0.06	0.07	0.05
Scup	0.17	0.30	—	0.14	0.31	0.32	—
Sea robin	Neg.	Trace	Trace	0.02	0.10	Trace	Trace
Puffer	—	0.23	—	—	0.12	0.21	Neg.
Toadfish	—	Neg.	Neg.	—	0.03	0.12	Trace

* Gall bladder and bile. Only three values averaged; one of these was unusually high (0.78), possibly due to contamination by pancreatic tissue.

The Puffer.—Proteases were not estimated in the puffer. Tables V and VI show that the amylase and lipase are correlated with the general activity of the fish.

The Toadfish.—In this, the most sluggish of the species studied, the amounts of digestive enzymes, except lipase, are the least. The large liver is apparently the chief source of enzymes (Tables IV, V, and VI).

DISCUSSION

Both Kenyon (1925) and Vonk (1928) have found that the herbivorous carp has much more amylase than have the carnivorous fishes. The present investigation shows that this does not apply in the case of the menhaden, which feeds largely upon small plants. This diet is rich in oils, yet the menhaden appears to have but slight quantities of lipase.

Tables IV, V, and VI show clearly that the amounts of digestive enzymes, in the fishes studied, are a correlative of the general activity. At one extreme are found the mackerel and menhaden—fishes that probably never cease swimming and thus impose constant demands upon their energy stores. At the other extreme is the toadfish, with the other fishes ranging in between.

As for the distribution of enzyme production among the various organs concerned with digestion, no generalization can be made because of the limited number of species studied. However, in the mackerel, which has a compact pancreas, the production of enzymes is centered for the most part in that organ, while in the other species studied the pancreas is not well defined and enzyme secretion is not localized in it. In the menhaden, where the pancreas is somewhat disseminated through the cæca, both protease and amylase production reside here to a greater degree than in any other portion of the system, while lipase is secreted in several places. Lipase secretion is characteristic of the highly organized pancreas. In other fishes, in which the pancreas is even more diffuse, there are decreasing stages of localization of secretory function in that gland. Apparently a high degree of morphological organization is the concomitant of a high degree of physiological organization.

In the mackerel, which has a well-developed pancreas, the cæca play a relatively minor rôle in the secretion of enzymes. In the menhaden, scup, and sea robin, where the pancreas does not show a high degree of morphological and physiological differentiation, the cæca seem to be important in this respect. This is of particular interest since the cæca are embryologically equivalent to the pancreas.

From a consideration of the data in hand, it is difficult to decide if there is an accumulation of enzymes during fasting, but the general impression is that there is no piling up of enzymes in the secretory cells.

It is interesting to note that the biles are usually active enzymatically. In the case of the scup, the presence of enzymes in the bile is readily comprehensible, as sections of the liver show scattered acini of pancreatic tissue, and the liver extracts are active. However, in the sea robin the liver extracts are almost wholly inactive, the liver sections show no pancreatic tissue, and yet the bile contains a protease and amylase. Unfortunately, I have no sections of the sea robin gall bladder.

With the exception of the puffer, a rough correlation is shown between the amounts of amylase in the digestive systems, and the blood-sugar levels. Inasmuch as both of these parallel, in general, the activities of the fishes, there may be no deeper significant relation between the two. Yet it seems probable that the efficacy of starch and glycogen digestion should condition the amount of carbohydrate taken into the body from the tract. Once in the system, part of the carbohydrate may go into storage as fat or glycogen, but part of it might well go toward maintaining the blood-sugar level. However, with the interconversion of foods and potential sources of glucose in the fat and protein, the fish could be more or less independent of its starch-digestive power.

Conclusions

The biliary and pancreatic amylases of several marine fishes have a critical optimum at pH 7.2, when the temperature is 35° C., NaCl 0.05 M, phosphate buffer 0.05 M, and the period of digestion is 30 minutes.

The concentration of NaCl required to activate amylase fully is higher for fish amylase than for mammalian; for fish amylase, it was found that 0.05 M NaCl is sufficient.

The rate of digestion of starch varies inversely as the concentration of enzyme, over a considerable range.

The digestive enzymes parallel, in quantity, the general activity of the fishes studied. This is especially true of trypsin, but not of pepsin.

The menhaden, which subsists chiefly upon plant forms, is not adapted in having little lipase, since the diet is rich in oils.

Lipase is least in quantity in the fishes having a fatty metabolism (insofar as they store large reserves of fat). These are the menhaden and the sea robin.

In the fishes studied, the pancreas varies from a compact organ to an exceedingly diffuse structure. Apparently the degree of morphological organization is a concomitant of the degree of physiological organization. The compact pancreas is the center of enzyme secretion; the diffuse organ produces only a small portion of the enzymes poured into the enteron.

When the pancreas is not highly organized, the pyloric cæca seem partially to supplant it in enzyme secretion.

I wish to express my sincere gratitude to Professor F. G. Hall, under whose direction this work was done. I also acknowledge my indebtedness to the United States Bureau of Fisheries for the facilities afforded for the experiments.

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EGG-TRINUCLEARITY IN HABROBRACON[1]

P. W. WHITING

(From the Carnegie Institution of Washington, Cold Spring Harbor, N. Y., and the Marine Biological Laboratory, Woods Hole)

MOSAICS FROM BINUCLEATE EGGS

In the parasitic wasp *Habrobracon juglandis* (Ashmead) specimens are occasionally found which are mosaic for various hereditary traits. The theory has been advanced (Whiting, P. W., 1924) that these mosaics develop from binucleate eggs in which two nuclei (oötids from one oöcyte) of different genetic constitution take part in cleavage. Data have recently been summarized with respect to various genes (Whiting, P. W., 1932*b*).

Three groups of mosaics may be distinguished with respect to sex—females, gynandromorphs, and males. Thus far only one mosaic female from a binucleate egg has been reported (Greb, 1933, p. 183). The mother was heterozygous for the factor aciform, *ac*, which affects the antennæ and the genitalia. The father was aciform. The mosaic was aciform on one side, wild-type on the other. It is supposed that both egg nuclei, one containing aciform, the other its normal allelomorph, were fertilized by sperm with the recessive factor aciform.

Many gynandromorphs have been reported, for the most part coming from binucleate eggs with one nucleus fertilized. Thus the male parts are haploid and matroclinous, the female parts diploid and biparental.

Mosaic males develop from unfertilized binucleate eggs laid by heterozygous mothers. Such males have previously been reported mosaic for a large number of different characters. Males mosaic for various other traits have been found recently among the offspring of females heterozygous for these traits. The new factors involved in male mosaicism are crescent, *cr* (eyes); droopy, *dr* (wings); gynoid, *gy* (antennæ and abdominal sclerites); honey, *ho* (body color); carrot, wh^c (eyes). The eye color differences in the mosaics involving the locus white were clear-cut without any modification or intergradation, both in the case of carrot vs. black (wild-type) and of carrot vs. white. The latter are produced from carrot-white compound females.

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[1] The material here reported has been found by students and independent investigators at the University of Pittsburgh and at Pennsylvania College for Women, Pittsburgh, Pa. Study of specimens and preparation of the manuscript was done for the most part at Woods Hole and Cold Spring Harbor. Technical assistance has been furnished by the Committee on Effects of Radiation on Living Organisms (National Research Council).

Males in *Habrobracon* usually develop from unfertilized eggs, but when the mother is mated to a closely related male some of the sons are biparental. This is shown by the fact that they inherit paternal traits as well as maternal. They are diploid in chromosome number and sterile or produce very few offspring. It was therefore to be expected that rarely a mosaic male would be produced instead of a gynandromorph from a fertilized binucleate egg. The fertilized nucleus might develop into diploid male parts instead of female while the unfertilized nucleus would develop into haploid male parts as in the gynandromorph. Such a haplo-diploid male mosaic was reported (Whiting, P. W., 1932*b*, pp. 305–306) resulting from a cross of an orange-eyed female by a closely related black-eyed male. The eyes showed both black and orange regions and the specimen produced many orange daughters which were highly fertile. A second haplo-diploid male mosaic (No. 675) has recently been found by R. L. Anderson. One eye was orange, one black. When this male was crossed with an orange-eyed female, many orange-eyed daughters were produced and also three black-eyed daughters. The orange daughters were of normal fertility, but the three black proved sterile although they lived for several days and fed on caterpillars. Thus the gonads as well as the eyes were mosaic with haploid (orange) and diploid (black) regions and the male bred in part as a normal haploid, in part as a nearly sterile biparental male.

Several males mosaic for honey body color were also mosaic for other factors affecting the same structures as are affected by honey. Thus No. 627 had left wings honey, right shot-veins; No. 660 had left legs honey, right stumpy; No. 662 had left wings wild-type, right honey shot-veins and left legs stumpy, right honey; No. 645 had left antenna long, right honey, and the wings all long.

Several males have been found, mosaic for various factors of Chromosome II. The genes located in this group with map distances are: crepe-wings, *cw*,—13—honey, *ho* (body-color)—22—cantaloup, *c* (eyes),—11—long, *l* (antennae, wings, legs),—2—narrow, *n* (wings), —25—defective, *d* (R4 wing-veins). No. 643 (found by Forrest W. Miller) was from a mother heterozygous (*c.l.d/cw.ho*) for five of these factors. Left antenna was honey, right wild-type; eyes were mosaic for cantaloup; wings were crepe-wings; some legs were honey and others wild-type. The mosaic was bred to wild-type females and 20 daughters were tested by breeding. Results showed that 16 of the sperm of the mosaic contained *cw.c.d* while 4 contained *cw.ho.d*. According to the theory of origin of the two genetically different cleavage nuclei from the same oöcyte, the division separating the two chromatids which remained in the egg from the two that passed into the first polar nucleus was reductional for *cw, l* and *d* but equational for *ho* and *c*. A triple crossover may have occurred between two strands, a double between the other two.

Egg binuclearity will explain the origin of the mosaics above cited since not more than two combinations of the various factors are evident in their bodies or by breeding test.

Mosaic Males from Trinucleate Eggs

The origin of several males (found by Raymond J. Greb) produced by virgin females heterozygous for honey and for stumpy cannot be explained by egg binuclearity since in each case the legs represent three of the four possible combinations of the maternal factors. As an example, legs of No. 659 (Figs. 1–6) are wild-type, stumpy and honey stumpy but none are stumpy alone. Other arrangements are represented in different specimens but none involving more than three of the four combinations of factors.

The first example of a mosaic male from a trinucleate egg was found by Kathryn A. Gilmore. A heterozygous female (*c.l/n.d*) produced a mosaic male (No. 511) (Fig. 9) with narrow wings on the left side while the right was long (antennæ, wings, legs), defective (R4 veins), cantaloup (eyes). Legs of the right side were long. The factor long shortens the wings especially toward the tip, which in the primary is curved ventrally. It also lengthens the segments of the antennæ and the legs. Four matings were made between mosaic male No. 511 and cantaloup females. The first produced cantaloup females only; the second (two days later) produced wild-type only; the third (four days later) and the fourth (two days later) cantaloup females only. Fourteen of the cantaloup daughters showed by breeding test that they carried long and defective, while 33 of the wild-type daughters carried no recessives except cantaloup from their mothers. The mosaic therefore bred in part as cantaloup, long, defective; in part as wild-type. Trinuclearity is involved in the genetic composition of this male. The right side of the body and the gonads in part represent a single crossover (between *n* and *d*) strand. The left wings represent the alternative strand. The gonads in part represent a single crossover (between *l* and *n*) strand.

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GENETIC STRATIFICATION OF GONADS

A point of interest to be noted in the breeding behavior of the mosaic male (No. 511) described above is genetic stratification of the gonads. Sperm were in the first mating, *c.l.d;* in the second wild-type; while in the third and fourth they were again *c.l.d.*

Previous breeding tests of males with mosaic gonads have shown that there may be a slight tendency toward stratification (Whiting, P. W., and Anna R. Whiting, 1927, p. 98) or the two kinds of sperm may be produced in approximately equal numbers throughout the life of the male (Whiting, P. W., 1928, p. 299).

Genetic stratification in the ovaries was suggested in connection with the mutation to vestigial (Whiting, P. W., 1932*a*, p. 24) in which case 50 non-vestigial were produced during the early life of the mother (in vials *a, b, c*); 12 non-vestigial and 12 vestigial subsequently (in vial *d*); and 7 non-vestigial finally (in vial *e*).

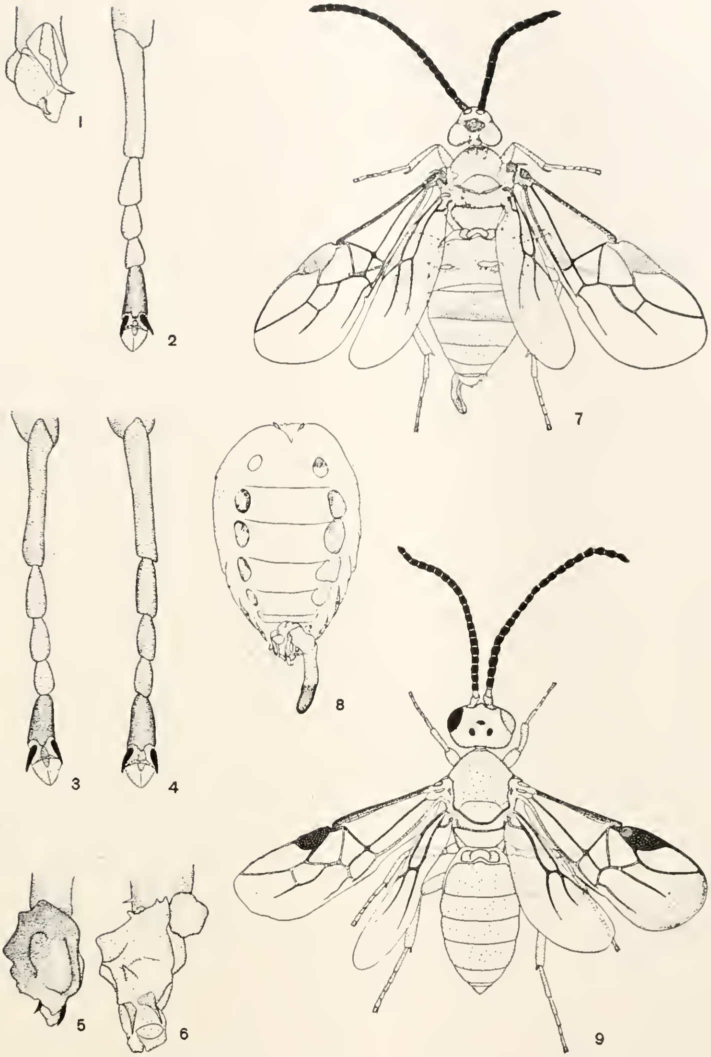
One ovary of the female producing the mutants attenuated (*Jour. Gen.,* in press) may have been stratified. In vials *a–c* there were 32/46 (69.56 per cent) attenuated, while in vials *d–j* there were 46/140 (32.86 per cent) attenuated. The difference is statistically significant, suggesting that posterior regions of both ovaries were heterozygous but that in anterior regions only one ovary contained the mutant factor.

In the case of a recent mutation, leglike antennæ (found by Raymond J. Greb), no leglike among 40 males occurred in vials *a–b*; 2 leglike among 19 males occurred in vial *c*; while 14 leglike among 22 males occurred in vials *d–e*.

A TRINUCLEATE GYNANDROMORPH

Gynoid, a factor for weak male intersexuality, increases sclerotization of the abdomen and shortens the antennæ of males, thus making them resemble females superficially. Gynoid females are indistinguishable in appearance from wild-type females.

In connection with studies on the nature of biparental males, Magnhild Torvik-Greb crossed gynoid females with eyeless males. Offspring were gynoid males and wild-type (biparental) males and females as expected. Gynoid is closely linked with eyeless (map distance 7). One fraternity contained in addition to these three expected classes a gynandromorph (No. 640) (Fig. 7) displaying three genetically distinct types of tissue. The head was typical eyeless with left antenna normal male. The right antenna was defective toward the tip showing considerable fusion of segments. The abdomen (Fig. 8) was female on the left side, gynoid male on the right except that the first right



Explanation of Plate I

Figs. 1–6. Tarsi of male No. 659, mosaic for honey and for stumpy. × 80.

Fig. 1. Left prothoracic, honey stumpy.

Fig. 2. Right prothoracic, wild-type.

Fig. 3. Left mesothoracic, wild-type.

Fig. 4. Right mesothoracic, wild-type.

Fig. 5. Left metathoracic, stumpy.

Fig. 6. Right metathoracic, honey stumpy.

Fig. 7. Dorsal view of gynandromorph No. 640, mosaic for eyeless and for gynoid. × 14.

Fig. 8. Ventral view of abdomen of gynandromorph No. 640. Note female, gynoid male, and wild-type male sternites. × 22.

Fig. 9. Dorsal view of male No. 511, mosaic for long (antennæ and wings), for narrow (wings) and for cantaloup (eyes). × 13.

sternite was characteristic for wild-type male, probably genetically eyeless like the head. Genitalia consisted of a complete male set and a large sensory gonapophysis (female) on the left.

The specimen may have started development as a female from a normally fertilized egg. Some sort of somatic segregation may then have given rise to the two types of male tissue, eyeless and gynoid.

An alternative hypothesis is that of egg binuclearity with fertilization of one nucleus to form the female parts and cleavage of a supernumerary sperm nucleus as well as of the unfertilized oötid to form the two types of male tissue. Development from a sperm nucleus (androgenesis or merogony) must be very rare if it occurs at all in *Habrobracon*. A gynandromorph (No. 523) produced by a black-eyed female (stock No. 25) crossed with an orange-eyed male (stock No. 3) had a male head with orange eyes. The recessive paternal trait appearing in the male parts may be explained by androgenesis or by somatic segregation. Among offspring from similar crosses made later with the same stocks there occurred a gynandromorph with male parts matroclinous (black eyes) as expected from the general rule. The question of androgenesis therefore remains open.

IMPATERNATE GYNANDROMORPHS

With only two exceptions all the gynandromorphs found in *Habrobracon,* amounting to over 150, have occurred in bisexual fraternities among the progeny of mated females. Whenever the female parts could show a paternal effect this was apparent, indicating that gynandromorphs come from fertilized eggs.

The two exceptional impaternate gynandromorphs (found by Kathryn A. Gilmore) occurred in the course of experiments in production of impaternate females. Females of a certain strain of Minnesota stock crossed with various males give rise to females which if bred as virgins occasionally produce a daughter. This is contrary to the usual rule of parthenogenetic production of males only, arrenotoky. Genetic analysis indicates that such daughters develop as a result of suppression of the second oöcyte division, in other words from a diploid nucleus. Impaternate gynandromorphs would be expected to occur in this material if in addition to a second oöcyte nucleus a reduced nucleus, an oötid from division of the first polar nucleus, took part in cleavage. An impaternate gynandromorph would then be comparable to a trinucleate haploid male mosaic except that one diploid nucleus failed to undergo reduction.

The first impaternate gynandromorph (No. 522) was found August, 1931, among the offspring from a virgin female heterozygous for tapering (antennæ) and for white (eyes), *ta/wh*. Left antenna was female (14 segments), right was male (21 segments). Eyes and ocelli were wild-type (black), the latter female (small). The abdomen was entirely female. Thorax was asymmetrical, being female (lighter in color) on the left side, male on the right. Since neither male nor female antenna nor male nor female regions of the eyes showed the mutant traits for which the mother was heterozygous, it may be supposed that the female parts were heterozygous for both genes, while the male parts carried both dominants.

The second impaternate gynandromorph was produced by a virgin female heterozygous for crescent (eyes). The head was male (23 segments in each antenna) with large black ocelli and normal eyes; the abdomen was female. Male parts were thus non-crescent but the character of female parts is indeterminable.

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A NEW TYPE OF SEX-INTERGRADE[1]

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Two types of sex-intergrades have been clearly distinguished (Goldschmidt, 1931) having certain regions of the body apparently male, other regions female. In gynandromorphs male and female regions differ from each other not only phenotypically but in genetic constitution as well. Intersexes, on the other hand, have all parts of the body of similar genetic make-up. Male and female regions appear phenotypically different but their chromosomal constitution is identical. Intersexes are classed as either male or female. Male intersexes begin their development as males but organs developed later are feminized. Female intersexes begin their development as females but organs developed later are masculinized.

A third type of sex-intergrade is reported in the present paper. These are genetically male mosaics with feminized genitalia. No genotypically female tissue is present, but the two types of male tissue interact to produce the feminization. Mosaic males with feminized genitalia resemble gynandromorphs and have for convenience been called gynandroid.

In the parasitic wasp, *Habrobracon juglandis* (Ashmead), normal (zygotic, diploid) females develop from fertilized eggs, normal (azygotic, haploid) males from unfertilized eggs. Diploid males of biparental inheritance (showing dominant traits of both parents) occur in small numbers from certain types of crosses. Such males have diploid sperm and are sterile or nearly sterile. When they have dominant factors and are crossed with recessive females, their few offspring are triploid females showing the dominant traits of their diploid father. There is no evidence of intersexuality in diploid males or in triploid females unless the reduced size of ovaries and near sterility of the latter indicate this. Moreover, their reproductive reactions are entirely normal, the diploid males mating readily and the triploid females stinging caterpillars and feeding and ovipositing upon them.

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[1] The material here reported has been found by students and independent investigators at the University of Pittsburgh and at Pennsylvania College for Women, Pittsburgh, Pa. Study of specimens and preparation of the manuscript was done for the most part at Woods Hole and Cold Spring Harbor. Technical assistance has been furnished by the Committee on Effects of Radiation on Living Organisms (National Research Council).

GYNANDROMORPHISM

Gynandromorphs occasionally appear in bisexual fraternities. Gynandromorphic offspring from a recessive female by a dominant male show the maternal trait in the male (haploid) parts, the paternal trait in the female (diploid) parts. This evidence is consistent with the theory of egg binuclearity. Fertilization of one nucleus results in the female parts; development of the other without fertilization results in the male parts. Gynandromorphs may have male heads, female abdomens, or the reverse; one side may be male, the other female; anterior left and posterior right may be of one sex, the remainder of the other sex; male islands may occur in female regions (Fig. 4) or female islands in male regions (Figs. 1 and 25). When genitalia are mixed in sex, there may be a full set of male structures and a half set of female structures. Two of these cases have been published (Whiting, P. W., and Anna R. Whiting, 1927. Page 114 and Plate I, Figs. 17–21) (Whiting, P. W., and Edward J. Wenstrup, 1932. Page 33 and Figs. 13*c* and 14). A third specimen of this type (No. 509) is female on the left side of the body (short antenna, larger wings, larger abdominal sternites), male on the right. The dorsal side of the abdomen (Fig. 2) is asymmetrical, showing male coloration on right side, female on left. The half set of female structures is placed laterally and anterior to the full male set (Fig. 3), a fact which may furnish cause for speculation as to homologies of parts.

WEAK MALE INTERSEXUALITY

A single mutant-type male found in a bisexual fraternity by Raymond J. Greb illustrates weak male intersexuality. Antennæ are short as in the female (Fig. 10) and abdominal tergites (Fig. 8) and sternites (Fig. 9), especially in the anterior part of the abdomen, are heavier than in the normal male, thus approaching the condition found in the female. Ocelli are large, of normal male size; genitalia are of normal male character. Instincts are typical for the male and mating readily takes place. The mutant-type called gynoid, *gy,* is of normal viability, segregating in approximately equal numbers among offspring from heterozygous mothers. Females heterozygous for gynoid have thus far produced five males mosaic for gynoid. Gynoid females have been derived by mating heterozygous females to gynoid males. They are structurally indistinguishable from wild-type females, are of normal viability and fertility and produce, as virgins, gynoid males only. A gynoid stock has been derived which breeds true.

Gynoid has been tested for linkage with many other genes. It proved to be closely linked with eyeless, *el.* Counts from heterozygous virgin females (*el/gy*) gave wild-type 112, eyeless 1157, gynoid 1484, eyeless gynoid 80; 6.7 per cent crossovers. When gynoid is crossed with non-gynoid, the biparental males are wild-type as is the case with any other recessive trait. Reciprocal crosses between gynoid and eyeless have been made by Magnhild M. Torvik. Ten eyeless females crossed with gynoid males produced wild-type females 296, wild-type (biparental) males 43, eyeless males 155. Sixty-five matings of the biparental males resulted in only nine females to 3372 males. These females were sterile. Twenty-three gynoid females (10 orange and 13 heterozygous for orange) crossed with eyeless males produced wild-type females 794, wild-type (biparental) males 30, gynoid males 435. None of the daughters or biparental sons were orange, while approximately 50 per cent of the gynoid sons from heterozygous mothers (+/o) and all of the gynoid sons from orange mothers were orange as expected. Twenty-four matings of the biparental sons resulted in no daughters and 1566 males.

Heredity of this factor for weak male intersexuality is therefore in every way similar to that of other recessives.

Feminized Genitalia in Mosaic Males

Virgin mothers heterozygous for various traits occasionally produce a son mosaic for one or more of these traits. These mosaic males develop from unfertilized eggs in which polar nuclei as well as the reduced egg nucleus presumably take part in parthenogenetic cleavage.

Normal male genitalia (Fig. 27) consist of a median penis on either side of which is an inner clasper made up of a basal part and a movable terminal part. Laterad to these is the pair of outer claspers surmounted by several bristles. The claspers are connected proximally with a tri-

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Explanation of Plate I

Fig. 1. Ventral view of abdomen of gynandromorph (No. 482), showing male genitalia and sternites with a female island indicated by a large sternite. × 22.

Fig. 2. Dorsal view of abdomen of gynandromorph (No. 509), showing pigmental asymmetry, female on left, male on right. × 22.

Fig. 3. Ventral view of genitalia of gynandromorph (No. 509) with complete male set posteriorly and female gonapophyses (sensory and two elements of sting) on left. × 80.

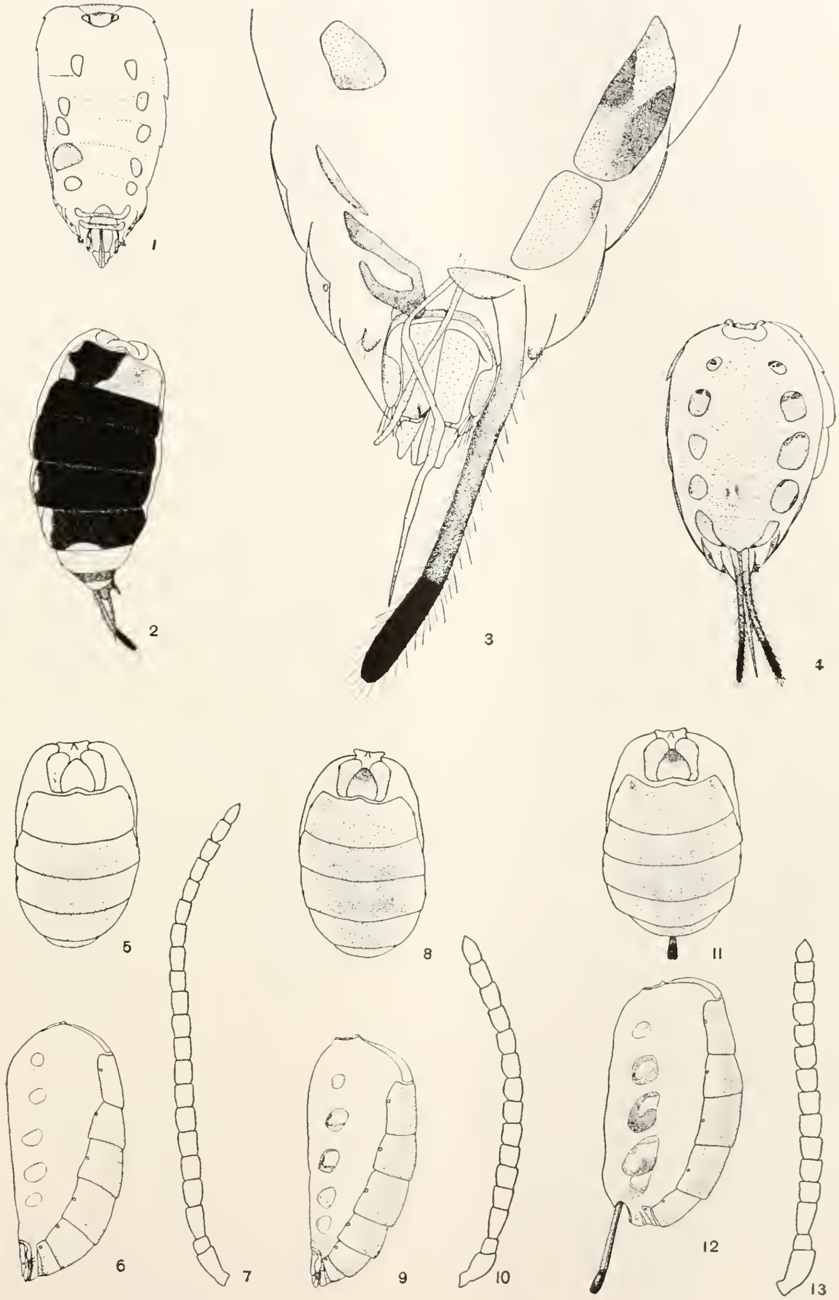
Fig. 4. Ventral view of abdomen of gynandromorph (No. 499), showing female genitalia and sternites with a male island indicated by two small sternites. × 22.

Figs. 5, 8, 11. Antero-dorsal views of abdomens of wild-type male, gynoid male, and female respectively. × 22.

Figs. 6, 9, 12. Lateral views of abdomens of wild-type male, gynoid male, and female respectively. × 22.

Figs. 7, 10, 13. Antennæ of wild-type male, gynoid male, and female respectively. × 33.

PLATE I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angular basal piece which may be withdrawn into what appears ventrally as the sixth and seventh abdominal segments. A pair of cerci surmounted by bristles are seen laterad to the genitalia.

Marked genital abnormalities are characteristic of certain mutant types, but occur rarely in other cultures. Among 299 mosaic males, however, there were 36 showing striking abnormality, including extreme displacement, reduplication or deficiency of parts, and feminization. Males showing the last trait have been called gynandroid because they suggest gynandromorphs. Genitalia of large numbers of non-mosaic siblings of mosaic males have been carefully examined and counts from miscellaneous cultures have often involved examination of genitalia more or less closely. Genital abnormalities in these are much less frequent than in their mosaic sibs.

No. 666 was a gynoid male mosaic for cantaloup eye color. Genitalia were almost normal (Fig. 29), but the right outer clasper was feminized laterally showing a small protuberance suggesting a female structure, a sensory gonapophysis. Posterior sternites were heavier on the right side, indicating feminization.

No. 678 was a gynoid male mosaic for cantaloup. Genitalia (Fig. 28) consist of a complete male set plus a sensory gonapophysis on the right (which conceals the right outer clasper in the drawing).

No. 652 was gynoid on the right side, wild-type on the left. Dorsal view of abdomen (Fig. 14) shows correspondingly heavier anterior

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EXPLANATION OF PLATE II

FIG. 14. Dorsal view of abdomen of mosaic male No. 652, showing pigmental asymmetry. × 22.

FIG. 15. Ventral view of mosaic male No. 652, showing gynoid antennæ and sternites on right. × 22.

FIG. 16. Genitalia of mosaic male No. 652, showing feminization and bilateral reduplication. × 80.

FIG. 17. Genitalia of mosaic male No. 579, showing feminization on right, deficiency on left. × 80.

FIG. 18. Genitalia of mosaic male No. 653, showing extreme feminization; sensory gonapophysis and sting on left. × 80.

FIG. 19. Dorsal view of abdomen of mosaic male No. 601, showing slight pigmental asymmetry. × 22.

FIG. 20. Ventral view of abdomen of mosaic male No. 601, showing darkening and thickening of sternites on right, feminization. × 22.

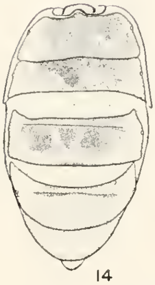
FIG. 21. Genitalia of mosaic male No. 601, showing feminization and reduplication on right. × 80.

FIG. 22. Genitalia of mosaic male No. 497, showing reduplication but no feminization. × 80.

FIG. 23. Genitalia of mosaic male No. 603, showing feminization and reduplication on right. × 80.

FIG. 24. Genitalia of mosaic male No. 659, showing reduplication but no feminization. Asymmetry in pigment is due to mosaicism for honey, *ho*, body color. × 80.

PLATE II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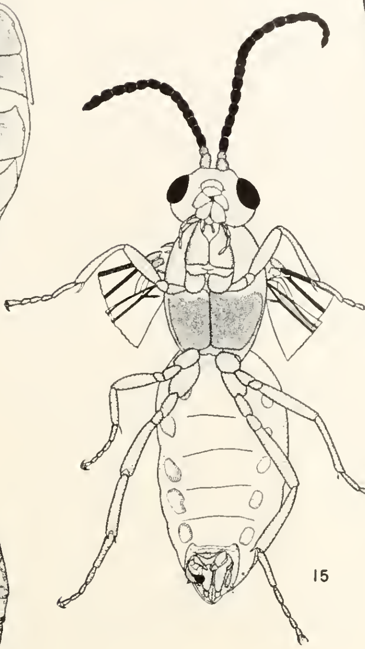
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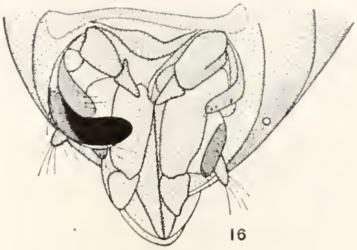
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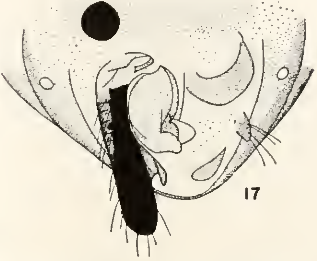
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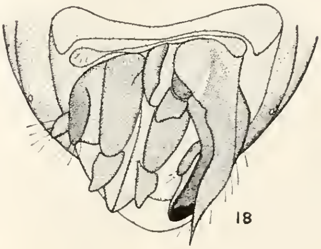
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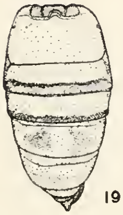
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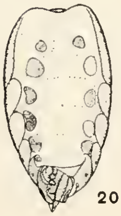
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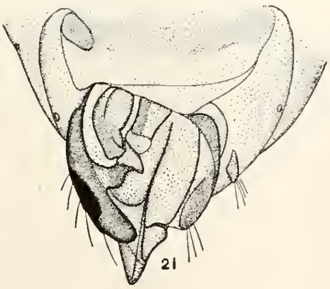
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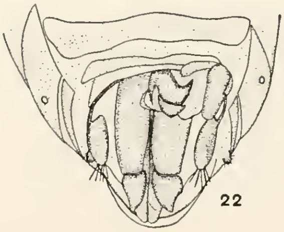
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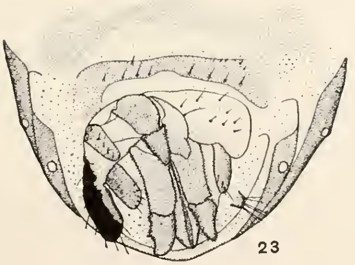
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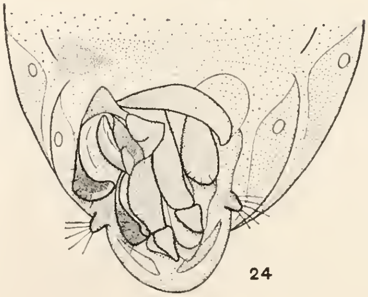
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tergites dextrally. Ventral view (Fig. 15) shows right abdominal sternites and right antenna characteristic for gynoid. Genitalia (Fig. 16) had sensory gonapophysis on right and complete reduplicated male set of genitalia cephalad to the terminal set.

No. 579 was mosaic for ivory eye color. Genitalia (Fig. 17) were lacking on the left. A well-developed sensory gonapophysis with a median lobe occurred on the right and also an inner clasper showing reduplication. Outer claspers were lacking.

No. 653 was mosaic for ivory, shot-veins and stumpy. Genitalia (Fig. 18) consisted of a complete male set with reduplicated male parts, a sting and a sensory gonapophysis on the left.

No. 601, mosaic for white eyes, showed pigmental asymmetry on dorsal side of abdomen (Fig. 19), as is frequent in mosaics. Enlargement of sternites is seen ventrally (Fig. 20) on the feminized right side. Genitalia (Fig. 21) showed sensory gonapophysis and reduplicated male parts on the right. The right inner clasper in the normal position lacked the terminal segment.

No. 603, mosaic for yellow antennæ and reduced wings, shows (Fig. 23) sensory gonapophysis and reduplicated male parts on right.

Reduplicated male parts occurring in mosaics which show no feminization are illustrated by No. 497 (Fig. 22) and No. 659 (Fig. 24). The latter male was mosaic for honey body color and it may be clearly seen that the line dividing honey from non-honey regions passes through the genitalia. The difference is especially well-marked in the outer claspers, left being honey and right (reduplicated) being non-honey.

Asymmetry in number of antennal segments has been shown (Whiting, P. W., 1932) to be correlated with male mosaicism. More or less asymmetry in growth of genitalia has been noted in several mosaic males in which there is neither reduplication nor deficiency of parts. Thus either the inner or the outer claspers may be of different size or may originate more anteriorly on one side than on the other. The entire anal region as indicated by the cerci may be shifted laterally so that one cer-

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Explanation of Plate III

Fig. 25. Ventral view of abdomen of gynandromorph No. 596, showing female island. × 22.

Fig. 26. Genitalia of gynandromorph No. 596, showing either female island or feminization of male genitalia. × 80.

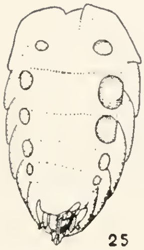
Fig. 27. Normal genitalia of mosaic male No. 647, showing gynoid abdominal sternites on right. × 80.

Fig. 28. Genitalia of mosaic male No. 678, showing feminization. × 80.

Fig. 29. Genitalia of mosaic male No. 666. The right outer clasper is definitely but slightly feminized. There is slight pigmental asymmetry of abdominal sternites posteriorly. × 80.

Fig. 30. Genitalia of mosaic male No. 664, placed transversely as if developed from gynoid (right) side. × 80.

PLATE III



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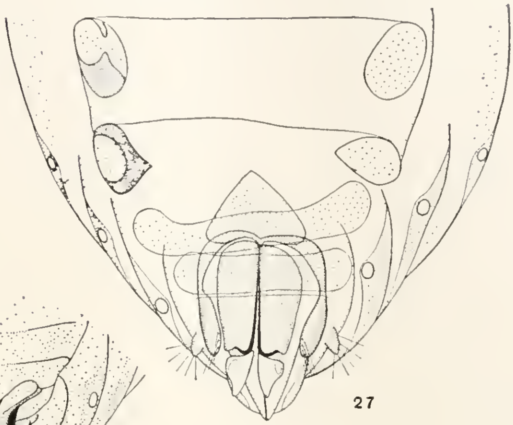

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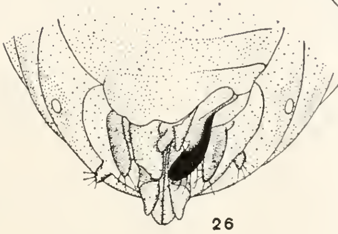

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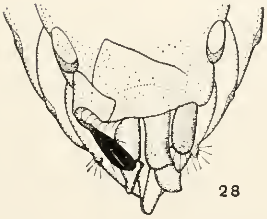

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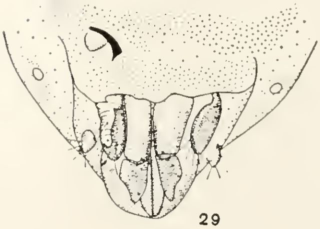

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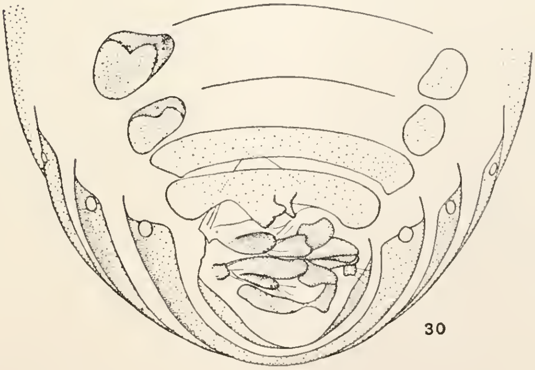

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cus is moved cephalad, the other caudad. A striking example of asymmetry in genitalia is illustrated by No. 664, a mosaic for gynoid and for cantaloup. The genitalia (Fig. 30) originate from the right side and extend toward the left. Right side is gynoid (note heavier sclerites, both sternites and tergites), left is non-gynoid. Difference in growth rate of the genetically different tissues may explain the anomaly. The sclerotized rudiments ventrad and cephalad to the transverse genitalia may represent deficient left claspers and half of penis. Structures of the right may therefore be reduplicated by a mirror image.

Genitalia of a mosaic male were figured (Whiting, P. W., 1928, Plate I, Fig. 2), showing a reduplicated right outer clasper and other structures suggesting an extra right inner clasper and penis. (This male was sterile, probably on account of inability to copulate.)

A male (No. 685) not obviously mosaic had a complete set of normal male genitalia and in addition a well-developed feminized gonapophysis to the left of the left outer clasper. This male proved sterile in four observed matings.

Another male (No. 693) not obviously mosaic had, in addition to the complete male set, an extra inner clasper toward the base at the left. There was no evidence of feminization.

Thirty-six cases of striking abnormality occurred among the 299 mosaic males. Three showed decided malformation or displacement but no feminization, reduplication, or deficiency. The remaining 33 included 22 feminized and 11 not feminized. Among the feminized 3 showed reduplication of male parts as well as deficiency, 13 showed reduplication only, and 3 showed deficiency only. Of those not feminized 8 showed reduplication only and 3 showed deficiency only.

The 23 males with feminized genitalia (including besides the 22 mosaics, No. 685 which was not obviously mosaic) were summarized with reference to the side on which deficiency or reduplication of male parts appeared. The summary (Table I) indicates that there is high correlation between feminization and reduplication with respect to the side affected. Of the 22 feminized on one side only, 15 showed reduplication on the same side only, while one showed reduplication on both sides. There appears to be no correlation between feminization and deficiency, for only one showed deficiency on the feminized side alone while five showed it on both.

Genital Abnormality and Genital Mosaicism

Since feminization and other abnormalities of genitalia are relatively frequent in mosaics, the question presents itself as to whether this condition may be due to the presence of genetically distinct tissues in the genital region. The majority of the mosaics bred lacked definite factor differences visibly affecting the abdomen. The following cases, however, may be cited involving the factors miniature (body size), honey (body color), and gynoid (which enlarges and thickens abdominal sclerites).

TABLE I

Classification of mosaic males with feminized genitalia according to side of body showing reduplication or deficiency in male genitalia.

Side of body showing		
Feminization	Reduplication	Deficiency
Right 14	Neither 4	Neither 2
		Both 2
	Right 9	Neither 7
		Both 2
	Both 1	Neither 1
Left 8	Neither 2	Neither 1
		Both 1
	Left 6	Neither 5
		Left 1
Both 1	Neither 1	Neither 1

No. 343 was entirely miniature on the left side, non-miniature on the right. The difference apparently extended through the genitalia, which were markedly deficient, consisting of a left outer clasper and penis only.

No. 664 was gynoid on right, non-gynoid on left in the region of the genitalia, which were transversely placed (Fig. 30).

No. 659 showed much reduplication. The genitalia were divided into honey and non-honey regions (Fig. 24).

No. 645 showed genitalia with right side honey, left non-honey and containing many reduplicated parts.

Nos. 660, 661, 662 each had a complete male set honey, but small sensory gonapophyses non-honey; No. 661 had reduplicated male parts honey while No. 662 had reduplicated male parts non-honey.

These cases show that abnormality may be associated with genetic diversity. However, No. 647 (Fig. 27) indicates genetic diversity of tissues but normal genitalia and No. 648 having the right outer clasper honey, left non-honey, and genitalia normal, proves that genitalia may be mixed without necessarily causing abnormality.

FEMINIZATION OF ABDOMINAL STERNITES

Mosaicism for honey body color may cause asymmetry of sternal pigmentation but not of sternal sclerotization, which is much greater in the female than in the male. Eighteen of the 23 males showing feminization of genitalia had abdominal sternites approximately symmetrical in size while five had marked asymmetry. In two of these this asymmetry was due to mosaicism for gynoid, the gynoid tissue being on the side showing the feminized gonapophysis in one case (No. 652, Fig. 15) but on the opposite side in the other (No. 665).

Attention has already been called to asymmetry in sternites of No. 601 (Fig. 20), which was non-gynoid, and to No. 666 (Fig. 29), which was completely gynoid. In the case of No. 656 also, the last two sternites were larger on the left side, which showed both feminization and reduplicated male elements. These three cases of sternal asymmetry not due to mosaicism for gynoid and with heavier sternites on the side showing feminized genitalia suggest that the cause for feminization of genitalia and of sternites may be the same.

GYNANDROMORPHS WITH MIXED GENITALIA

Gynandromorphs with mixed genitalia have been found. Three (Nos. 5, 446, 509) had well-developed sensory gonapophyses and one or two elements of the sting on one side with a complete male set of genitalia terminally (Fig. 3).

A fourth gynandromorph (No. 673) was female on the left side of the body, male on the right. There were no external male genitalia, but on the left side were a long sensory gonapophysis and two elements of the sting, one of which was very long and thin.

A fifth gynandromorph (No. 596) was in general female on the left side, male on the right, showing much asymmetry of color. This asymmetry extended into the abdomen both dorsally and ventrally, the fifth tergite showing the sex difference. Ventrally the asymmetry extended through the third sternite, but posterior to that sternites were male (Fig. 25). Genitalia (Fig. 26) were male except for a small sensory gonapophysis and some minute rudiments of the sting or reduplicated parts.

A sixth gynandromorph (No. 689), which was anteriorly female, showed asymmetry in wings and in abdominal sclerites, the left side being female, the right male. Genitalia were very deficient, showing a minute sensory gonapophysis on the left and a small median structure which was possibly a penis.

The possibility should be mentioned that the last two gynandromorphs may have been also gynandroid with feminized male rather than female elements in the genitalia. In this connection should be recalled the male genitalia of a fertile gynandromorph previously reported, No. 395 (Whiting, P. W., and E. J. Wenstrup, 1932, p. 36), with a reduplicated outer clasper on the right, female, side.

FEMINIZED GENITALIA IN HABROBRACON BREVICORNIS (WES.)

"Intersexual males" of *Habrobracon brevicornis* (Wes.), a species parasitic on the European corn-borer, *Pyrausta nubilalis* Hübn., have been reported (Whiting, P. W., and Anna R. Whiting, 1927. Pages 112, 113, 115, Plate II) having in addition to the normal male set of genitalia a small "female" gonapophysis. One (No. 275) with very irregular asymmetrical abdominal sternites and short antennæ occurred in a bisexual fraternity containing also a gynandromorph and three males (?) with short antennæ and asymmetrical sclerites. Since this fraternity contained females, it is possible that the "intersexual males" may have been gynandromorphs.

A second, No. 193, occurring in a male fraternity and hence probably from an unfertilized egg, had antennæ of normal male length, abdominal sternites typical for the male, but with a small "female" gonapophysis located, as in No. 275, on the right side at the base of the normal male set of genitalia.

A previously unreported male of *H. brevicornis* had, in addition to a normal male set of genitalia at the tip of the abdomen, a second complete set on the left side near the base of the typical set. The left external clasper of this extra set was somewhat thickened and of slightly darker color than normal while its right external clasper was black and small with cross lines suggesting a female sensory gonapophysis.

Thus it is suggested that a condition similar to gynandroid may occur in *H. brevicornis*. No factor differences indicating mosaicism were available, however.

DISCUSSION

The theory of sex-determination in Hymenoptera recently advanced (Whiting, P. W., 1933. *Biol. Bull.*, **65**: 357) was first suggested by feminization of genitalia in mosaic males. It was postulated that the mosaic is composed of two types of tissue each recessive for a different sex factor, the dominant allelomorphs to both of which are necessary for femaleness. One type of tissue, X, contains sex factors F and g, while the other, Y, contains their allelomorphs f and G. Females $X + Y$ are diheterozygous, $F.g/f.G$, and normally produce two types of males, X and Y, genetically different but phenotypically similar.

The analogy was drawn with mosaicism for eye color. A male may have eyes mosaic for two recessive whites (white, *wh*, and ivory, o^i) genetically different but phenotypically similar. The dominant allelomorph to ivory present in the white region apparently causes the production of some substance that diffuses through and interacts with the dominant allelomorph to white, producing black pigment at the border in the ivory region. No diffusion seems to take place in the reverse direction. Similarly in feminized mosaics factor G in the Y region of the body causes some substance that diffuses through and interacts with factor F in the X region, producing female-like structures at the border in the X region.

It is to be noted that reduplication of male genitalia is relatively frequent in mosaics and is highly correlated with the side showing feminization. It may likewise be recalled that in the less pronounced cases it is the outer clasper that shows feminization. These facts suggest that the gonapophysis is a reduplicated and modified outer clasper and that reduplication is one phase of feminization. Thus it may be that the eight mosaics showing reduplication but no feminization are like those showing feminization in that they are composed of X and Y tissue with the reduplicated structures on the X side.

Not all genital irregularities in mosaic males, however, are to be ascribed to interaction of X and Y. Deficiency of parts has no correlation with the side feminized and many types of distortion may be due merely to genetically diverse growth rates, as in the case of antennal asymmetry.

Normality of genitalia in mosaics is to be ascribed in the majority of cases to non-mosaicism of the genital region or, in those cases in which the genitalia are mosaic, to the fact that both regions are X or both Y. Mosaicism would then not involve the sex factors but other differences.

The rare cases of reduplication or feminization occurring in males not obviously mosaic may be ascribed to origin from binucleate eggs in which no visible genetic difference was involved or to failure of bodily structures to exhibit the genetic mosaicism.

Mixed genitalia in gynandromorphs are usually attributable to the presence of both female ($X + Y$) and male (X or Y) tissue. It is, however, possible that some cases arise from trinucleate eggs in which one nucleus was fertilized while the other two (X and Y) gave rise to genetically diverse male tissue and gynandroid genitalia.

Feminization of abdominal sternites is less frequent than that of genitalia but the three cases cited were on the same side of the body as the feminized genitalia. Asymmetry of ocelli has been observed in some mosaic males but this trait is too variable for definite conclusions. The markedly increased antennal asymmetry of mosaic males cannot be ascribed to a feminizing influence. No shortening of antennæ suggesting feminization has been found in mosaic males, nor is there any feminization of instincts. Preliminary survey of gonads and of other internal structures has as yet yielded no evidence of feminization.

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BIPARENTAL MALES AND BIPARENTAL RATIOS IN HABROBRACON

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Genetic evidence for the diploid nature of biparental males in the parasitic wasp, *Habrobracon juglandis* (Ashmead), has been reported by Anna R. Whiting (1927) and Magnhild M. Torvik (1931). It was demonstrated that biparental males were diploid for the seventeen mutant factors tested, and no evidence was found to indicate that they were simplex for a sex chromosome, as had been suggested. Magnhild M. Torvik showed cytologically that biparental males have twice as many spermatogonial chromosomes as occur in azygotic males. P. W. Whiting and R. L. Anderson (1932) reported evidence inconsistent with the theory that the loss of a sex chromosome in gametogenesis causes fertilized eggs to develop into males rather than females. The percentage (or ratio) of males among biparentals (biparental males × 100/biparentals) was shown to be increased with increase of temperature or degree of relationship and to be negatively correlated with proportion of biparentals (biparentals × 100/total). They concluded that some condition arising at fertilization or subsequent to it determines the sex of biparentals.

In the investigations reported at this time these two percentages were studied in four experiments. In one a successful attempt was made to increase the proportion of biparental males by selection. In the second experiment orange eye-color from stock No. 3 was graded up to wild-type stock No. 11, giving females having various proportions of the original material from Nos. 3 and 11. A similar experiment was carried out for another orange-eyed stock, *D-o,* and wild-type stock No. 25, and a fourth which involved *D-o* and wild-type stock No. 31.

The origin of the stocks used will be described in the account of the separate experiments. In the making of crosses matings were observed and each male was left with the female from four to six hours. The females were then placed singly in shell vials and fed four or five host caterpillars, and the vial termed *a*. Four days later each female was transferred to a new vial, marked *b*, and fed again. Transfers were made in turn every four days until the females had been in vials *e* for four days. At that time the females were discarded, since they tend to exhaust their supply of sperm shortly after that time and thenceforth give rise to azygotic males only. The cultures were kept in incubators regulated for a constant temperature of 30° C., except for short intervals during which transfers were made from one vial to another.

The author is indebted to Professor P. W. Whiting, at whose suggestion the investigation was begun, for guidance during the progress of the work, and to Professor Anna R. Whiting for advice and criticism. Acknowledgment is also made to the Committee on Effects of Radiation on Living Organisms of the National Research Council for technical assistance through grants to Professor P. W. Whiting.

THE INFLUENCE OF SELECTION

An experiment designed to show the effect of selection on the percentage of males among biparentals and on the percentage of fraternities including such males was carried through eleven generations by D. R. Charles, and from that point through the thirty-first generation by the author.

The experiment was begun by crossing orange-eyed females (stock 19*c*) to black-eyed males (wild-type stock No. 1). The offspring included orange-eyed azygotic males (*o*), a few black-eyed biparental males (*O*), and black-eyed females (*Oo*). Reference to Table I will show that five of the sixty fraternities contained biparental males. The heterozygous females, *Oo,* which were sisters to biparental males, were allowed to mate with their azygotic brothers, *o*. The F_2 generation included then *Oo* and *oo* females, and *O* and *o* males. Biparental males could not be detected in this generation. To produce the F_3, *oo* females were mated to their *O* brothers. Five of the 34 fraternities contained biparental males. The F_4 was produced in the same way as the F_2, and the F_5 as the F_3. The strain was continued in this way, always by brother-sister matings, through the F_{21} generation. It will be seen that the odd-numbered generations contained biparental males which could be distinguished from azygotic males, and that the even generations were used in continuing the strain.

Considering all the fraternities in each of the odd generations (Table I), there is to be seen a steady rise in the percentage of males among biparentals up to the ninth generation, from which time the value is more or less constant, except for small fluctuations. The drop from 10 per cent of the F_9 to 6.5 of the F_{11} (difference, 3.5 ± 1.2 [1]), may have been due to chance selection or to some unknown environmental factor. The

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[1] Standard errors are used throughout this paper.

Table I

Selection Experiment

Generation	Number of matings	Percentage with +♂♂	o ♂♂	+♂♂	+♀♀	$\frac{\text{Biparental males} \times 100}{\text{Biparentals}}$	$\frac{\text{Biparentals} \times 100}{\text{Total}}$
F_1	5		32	7	32	17.9±6.1	53.9±5.9
	55		477	0	547		53.4±1.6
Total	60	8.3	509	7	579	1.2± .4	53.5±1.5
F_3	5		44	6	20	23.1±8.3	37.1±5.8
	29		175	0	151		46.3±2.8
Total	34	14.7	219	6	171	3.4±1.3	44.7±2.5
F_5	20		162	36	246	12.8±2.0	63.5±2.3
	16		93	0	123		56.9±3.8
Total	36	55.6	255	36	369	8.9±1.4	61.4±1.9
F_7	48		532	77	606	11.3±1.2	56.2±1.4
	31		307	0	282		47.8±2.1
Total	79	60.8	839	77	888	8.0± .9	53.5±1.1
F_9	63		1022	152	1109	12.1± .9	55.2±1.0
	24		236	0	259		53.2±2.2
Total	87	72.4	1258	152	1368	10.0± .8	54.7± .9
F_{11}	126		3372	356	4400	7.5± .4	58.5± .6
	32		708	0	739		51.1±1.3
Total	158	79.7	4080	356	5139	6.5± .3	57.3± .5
F_{13}	36		1082	145	1130	11.4± .9	53.7±1.0
	4		132	0	84		38.8±3.3
Total	40	92.5	1214	145	1214	10.7± .8	52.4±1.0
F_{15}	12		298	25	391	6.0±1.1	58.2±2.2
	5		136	0	141		50.9±3.0
Total	17	71.6	434	25	532	4.5± .9	56.2±1.8
F_{17}	10	100.	106	22	226	8.9±1.8	70.1±2.4
F_{19}	16		625	55	529	9.4±1.2	48.3±1.4
	1		97	0	32		24.8±3.7
Total	17	94.1	722	55	561	8.9±1.1	46.0±1.3
F_{21}	15		415	59	434	11.9±1.4	54.3±1.6
	3		61	0	84		57.9±4.0
Total	18	83.3	476	59	518	10.2±1.2	55.0±1.5

F_{15} generation was bred when an incubator was not available and the low percentage may have been due to the variable temperature or to chance selection. Percentages for the seventeenth, nineteenth, and twenty-first generations are about the same, indicating that no further increase by selection was being obtained.

If one considers only those fraternities including biparental males, the percentage of males among biparentals shows no significant rise or deviation from one generation to another. Obviously then the increase in the percentage for all fraternities is due to an increase in the percentage of matings producing biparental males. These percentages show a steady rise up through the F_{13} generation, after which they fluctuate around an average of 85 or 90.

Since the ratios of males among biparentals were practically the same for the seventeenth, nineteenth, and twenty-first generations, selection seemed no longer effective. To determine whether or not the strain had become genetically homogeneous through selection and close inbreeding (brother-sister matings), parents for generations 23 through 31 were selected on a different basis than through 21. Through the F_{21} generation orange females and black males, which were crossed to produce biparental males, were always offspring of black, heterozygous females which were sisters to biparental males. From the F_{21} generation the parents of the odd generations were chosen from fraternities, of the preceding odd generation, having no biparental males or having a percentage termed high, medium, or low. Percentages from 0–7 were termed low, from 7–14, medium, and from 14 up, high.

In the F_{23} generation there were three groups of fraternities (Table II, column 2, "basis of selection") whose parents were derived from fraternities having, respectively, average percentages of 17.1, 9.5, and zero. All three groups had, however, approximately the same ratio. The same results were obtained in the F_{25} generation but in the F_{27} chance selection was apparently effective in an opposite direction. Parents from several fraternities having an average percentage of 9.8, medium, gave offspring showing a percentage of 3.8 ± 1.07. Parents from fraternities having a low percentage, averaging 6.3, gave offspring showing a value of 19.1 ± 2.25. The difference between the two is very significant from the statistical standpoint, being 15.3 ± 2.49. The cause of this difference may have been due, in addition to the factor of chance, to the occurrence of one or more mutations affecting the production of biparental males.

Table I shows the ratios of biparentals for fraternities including biparental males and for those without them. No significant difference exists between the two groups of fraternities in generations 1, 3, 5, 9.

15, or 21, while in the other generations the fraternities including biparental males have significantly higher ratios of biparentals. In one of these, however, only a single fraternity lacked biparental males, and the low biparental ratio of a single fraternity may be due to fertilization by a male below the average in fertility. The differences are really significant in generations 7, 11, and 13. The meaning of this will be discussed later when comparisons are made with other experiments.

Table II

Selection Experiment: Results of Selection in Several Directions

Generation	Basis of selection	Frats. with biparental* ♂♂ / Total frats.	o ♂♂	+ ♂♂	+ ♀♀	Biparental males × 100 / Biparentals	Biparentals × 100 / Total
F_{23}	17.1	12/12	367	46	379	10.8	53.6
	9.5	11/12	424	44	386	10.2	50.3
	none	10/10	288	30	325	8.5	55.2
Total		33/34	1079	120	1090	9.8	53.3
F_{25}	16.2	9/12	354	21	347	5.7	50.8
	12.0	12/13	438	25	446	5.3	50.7
	5.1	12/15	563	24	448	5.1	45.6
Total		33/40	1345	70	1241	5.3	49.3
F_{27}	9.8	7/13	403	12	302	3.8	43.8
	6.3	6/6	128	30	127	19.1	55.1
Total		13/19	531	42	429	8.9	47.0
F_{29}	20.0	2/2	23	4	38	9.5	64.6
	none	6/7	66	16	86	15.6	60.9
Total		8/9	89	20	124	13.8	62.2
F_{31}	20.0	3/3	107	6	116	4.9	53.3
	9.4	10/10	357	36	396	8.3	54.7
	none	4/4	141	8	201	3.8	59.5
Total		17/17	605	50	713	6.5	55.8

* Parents were selected from several fraternities whose parents belonged to fraternities with the average ratio of biparental males indicated.

The Effect of Grading

The 11-o Experiment

Matings of orange-eyed females of stock No. 3 to black-eyed males of wild-type stock No. 11 do not produce biparental males, since the stocks are not related. The *Oo* daughters of such matings were crossed to their *o* sons or nephews, and *oo* females obtained. These *oo* females may be thought of as being composed of one-half stock No. 3 material and one-half stock No. 11 material. When 44 of these were mated to males of stock No. 11, biparental males were present among the offspring. Orange was continually graded up to stock No. 11. Females (*oo*), having less and less of the genetic material of stock No. 3, except the eye-color, were crossed to males of stock No. 11. The females used in the eighth series of matings were 3/512 stock No. 3 and 509/512 stock No. 11.

Considering first all fraternities for each series (Table III), one can see a gradual rise in the percentage of males among biparentals. The highest point was reached in the fifth series, the percentage being 10.6 ± 1.8 greater than in the sixth. In the seventh and eighth the percentage went up again, being 21.6 in the eighth. The drop from the fifth to the sixth was probably due to unconscious selection of hereditary factors.

If one considers the fraternities in each series which included biparental males, it is seen that the percentage begins as 9.1 and jumps to 17.0 in the second series. There is a gradual rise until the fifth, when the highest point, 29.0, is reached. Then follows a gradual drop until the value is 21.6 in the eighth.

Grading of stock No. 3 up to stock No. 11 was successful not only in increasing the proportion of fraternities containing biparental males but also in raising the percentage among those fraternities. Comparison of the two groups of fraternities also leads one to believe that two different kinds of hereditary factors regulating the production of biparental males are present. One kind allows such males to be produced, and another kind modifies their ratio. From the fifth to the sixth series there was a considerable drop in the percentage for all fraternities, but a much smaller drop among those fraternities containing biparental males. This suggests that there was unintentional selection against the factors which cause the production of these males, but no selection against the modifying factors.

Comparison of biparental ratios for fraternities with and without biparental males shows the ratio to be less in every series for those fraternities having biparental males. The difference is highly signifi-

Table III

11–o Experiment

Composition of *oo* ♀♀	Number of matings	Percentage with +♂♂	*o* ♂♂	+♂♂	+♀♀	Biparental males × 100 / Biparentals	Biparentals × 100 / Total
1/2 No. 3	17		252	28	280	9.1±1.6	55.0±2.1
	27		277	0	645		70.1±1.5
Total	44	38.6	529	28	925	2.9± .5	60.2±1.2
1/4 No. 3	11		167	39	190	17.0±2.5	57.9±2.5
	18		185	0	448		70.8±1.8
Total	29	37.9	352	39	638	5.8± .9	65.8±1.5
1/8 No. 3	17		541	254	725	25.9±1.4	64.4±1.2
	11		174	0	605		77.6±1.5
Total	28	60.7	715	254	1330	16.0± .9	68.8±1.0
1/16 No. 3	7		173	85	226	27.3±2.5	64.3±2.5
	2		71	0	179		71.6±2.8
Total	9	77.7	244	85	405	17.3±1.7	66.6±1.7
1/32 No. 3	22		639	362	885	29.0±1.3	66.1±1.1
	2		31	0	120		79.5±2.3
Total	24	91.6	670	362	1005	26.5±1.2	67.1±1.0
1/64 No. 3	11		306	101	281	26.4±2.2	55.2±1.9
	6		107	0	251		70.1±2.4
Total	17	65.7	413	101	532	15.9±1.4	60.5±1.5
1/128 No. 3	16		321	107	402	21.0±1.8	61.3±1.7
	2		14	0	52		78.8±5.0
Total	18	88.8	335	107	454	19.1±1.7	62.6±1.6
3/512 No. 3	25	100.0	1156	369	1339	21.6±1.0	59.6± .9

cant, except in one series, the fourth, where the difference is over twice the standard error. This negative correlation suggests that fewer biparentals are produced when biparental males occur.

The 25-o Experiment

An experiment was begun by mating 12 females of *D-o* stock to males of wild-type stock No. 25, collected originally in New York City and inbred for a number of years. *D-o* is an orange-eyed stock derived from the selection experiment described above. The 12 matings produced 411 orange males and 1067 black females. As expected, no biparental males were obtained, since the two stocks were unrelated. Some of the daughters, *Oo,* were mated to their *o* sons or nephews, and produced *Oo* and *oo* daughters. Twenty-three of the *oo* females, in composition one-half *D-o* and one-half stock No. 25, were crossed to males of 25, and biparental males were present among the offspring. By continual breeding up to 25, *oo* females were obtained which were in turn 1/4, 1/8, 1/16, and 1/32 *D-o*. Females of each group were crossed to males of stock No. 25 (Table IV).

TABLE IV

25-o Experiment

Composition of *oo* ♀♀	Number of matings	Percentage with +♂♂	*o* ♂♂	+♂♂	+♀♀	Biparental males × 100 / Biparentals	Biparentals × 100 / Total
1/2 *D-o*	7		263	55	396	12.2±1.5	63.2±1.8
	16		516	0	1291		71.4±1.1
Total	23	30.4	779	55	1687	3.2± .4	70.9± .9
1/4 *D-o*	5		151	11	172	6.0±1.7	54.8±2.7
	11		274	0	679		71.2±1.5
Total	16	31.2	425	11	851	1.3± .4	67.0±1.3
1/8 *D-o*	11		149	22	290	7.0±1.4	67.6±2.2
	13		222	0	528		70.4±1 6
Total	24	45.9	371	22	818	2.6± .5	69.4±1.3
1/16 *D-o*	15		596	121	868	12.2±1.0	62.4±1.2
	9		359	0	789		68.8±1.4
Total	24	62.6	955	121	1657	6.8± .6	65.1± .9
1/32 *D-o*	20		712	104	790	11.6±1.1	55.7±1.2
	4		141	0	368		73.2±2.0
Total	24	83.2	853	104	1158	8.2± .7	59.7±1.1

Considering all fraternities, the percentage of males among biparentals was 3.2 in the first series, followed by a small drop to the second, and thereafter a gradual increase, until in the fifth series the percentage was 8.2. The very small difference between that and the preceding suggested that further grading could not materially have increased the production of biparental males.

Consideration of fraternities containing biparental males shows a similarity to the 11-*o* experiment. In the initial crosses the percentage was 12.2 ± 1.5 as compared to 3.2 ± .42 for all fraternities. In the second and third series the value fell to 6.0 and 7.0, respectively, and rose again to 12.2 and 11.6 in the fourth and fifth. Since the percentage was no greater in the fifth than in the first, it suggests that in this experiment factors were present allowing the reaction to take place necessary for the production of biparental males, but that modifying factors were not present. In the 11-*o* experiment both kinds of factors were present, while the modifying factors were not present or of little importance in the selection experiment.

TABLE V

31-o Experiment

Composition of *oo* ♀♀	Number of matings	Percentage with +♂♂	*o* ♂♂	+♂♂	+♀♀	$\frac{\text{Biparental males} \times 100}{\text{Biparentals}}$	$\frac{\text{Biparentals} \times 100}{\text{Total}}$
1/2 *D-o*	9		264	53	375	12.4±1.6	61.8±1.8
	19		434	0	1236		74.0±1.1
Total	28	32.1	698	53	1611	3.2± .4	70.4± .9
1/4 *D-o*	15		569	156	463	25.2±1.7	52.1±1.4
	1		17	0	37		68.5±6.3
Total	16	93.7	586	156	500	23.8±1.6	52.9±1.4
1/8 *D-o*	16		350	90	390	18.7±1.8	57.8±1.7
	4		115	0	184		61.5±2.8
Total	20	80.0	465	90	574	13.5±1.3	58.3±1.4
1/16 *D-o*	4		112	72	155	31.7±3.1	66.9±2.5
	6		53	0	169		76.1±2.8
Total	10	40.0	165	72	324	18.2±1.9	70.6±1.9
1/32 *D-o*	17		593	141	463	23.3±1.7	50.5±1.4
	6		318	0	485		60.4±1.7
Total	23	73.8	911	141	948	12.9±1.0	54.4±1.1

As in the 11-*o* experiment, the percentage of biparentals is less in every case for fraternities including biparental males. The difference is highly significant except in one series, where it is 2.8 ± 2.7.

The 31-o Experiment

An experiment was begun by crossing 10 females of *D-o* stock to males of wild-type stock No. 31, taken in Pittsburgh, Pennsylvania, in 1929 and inbred since that time. The 10 matings produced 316 orange males and 724 black females. In the same manner as for the 25-*o* experiment, this line was bred up to males of 31, *oo* females being in turn 1/2, 1/4, 1/8, 1/16, and 1/32 *D-o*. Females of each group were crossed to males of stock No. 31 (Table V).

The results of the 31-*o* experiment are more similar to those of the 11-*o* experiment than to those of the 25-*o* experiment. As regards all fraternities, the percentage of males among biparentals happened to be the same in the first cross as it was in the 25-*o* experiment. There is, however, a very significant rise from 3.2 to 23.8 in the second series of crosses. In the third series the value was significantly less, in the fourth it went up again, and in the fifth down. These fluctuations must have been due to chance selection of factors regulating the number of such males produced. Study of the fraternities containing biparental males suggests that such modifying factors are present, as in the 11-*o* experiment. Differences between the 25-*o* and 31-*o* experiments are probably due to the differences in residual heredity of the two type stocks, since females of the same stock were used to initiate the two experiments.

It is interesting to note that for the selection experiment, from which the *D-o* stock was derived, the highest percentage of males among biparentals was 13.8 (F_{29}). The highest percentage for the 25-*o* experiment was 8.2 and for the 31-*o* experiment 23.8. This indicates further a difference between stocks Nos. 25 and 31 in regard to factors regulating male biparentalism.

As in the 11-*o* and 25-*o* experiments, the percentage of biparentals is less in every generation for those fraternities including biparental males. The difference is significant in every case except one, in which it is 3.7 ± 3.3.

THE INFLUENCE OF AGE OF MOTHER

In 1930 D. R. Charles reported that the percentages of males among biparentals decrease with increasing age of mothers. Percentages were calculated for the culture vials through which each female was successively passed, and were as follows: (*a*) 11.96 ± .34; (*b*) 7.71 ± .57;

(*c*) 7.77 ± .73; and (*d*) 5.41 ± .75. The two significant drops, from *a* to *b*, and from *a* to *d*, are 4.25 ± .67 and 6.55 ± .82, respectively.

Charles summarized data for the odd generations of the selection experiment through the eleventh generation. A summary by vials for generations 13, 19, 21, 23, 25, 27, and 31 is included in Table VI. Numbers for separate generations were too small to be significant, but the summary for all generations combined shows a decrease from vials *a* to *d*, and a very small rise in *e*. The significant drops are from *a* to *c*, 5.0 ± 1.1; from *a* to *d*, 6.2 ± 1.1; from *a* to *e*, 5.4 ± 1.5; and from *b* to *d*, 3.4 ± 1.1.

The 11-*o*, 25-*o*, and 31-*o* experiments were summarized in the same way, the data being recorded in Table VI. Separate generations indicated no decrease in the percentage with age of mother, and all generations combined, where the numbers are large enough to be significant, showed no difference from one vial to another. Actually, in the 11-*o* and 25-*o* experiments, vials *d* have the largest ratio, but the value does not differ significantly from any other.

Table VI

Percentages of Males among Biparentals for Successive Periods in the Life of Females

Experiments	Vials				
	a	*b*	*c*	*d*	*e*
Selection	12.6 ± .79	9.8 ± .78	7.6 ± .7	6.4 ± .8	7.2 ± 1.3
11-*o*	24.1 ± 1.2	22.6 ± 1.1	22.9 ± 1.3	26.1 ± 1.3	23.8 ± 1.6
25-*o*	14.7 ± 1.4	9.8 ± 1.1	10.4 ± 1.2	10.6 ± 1.3	9.5 ± 1.4
31-*o*	21.5 ± 1.7	21.5 ± 1.6	21.4 ± 1.7	25.1 ± 2.3	18.1 ± 2.6

No explanation will be offered at this time for the fact that in one experiment the percentage of males among biparentals decreases with increasing age of mothers, and in three other experiments percentages do not differ from one set of vials to another.

The 11-*o* experiment was also summarized to show the variations in the percentage of biparentals among total offspring with increasing age of mothers. Since the proportion of biparentals depends partly on the number of sperms received at mating, it was to be expected that this ratio would decrease with increasing age of the mothers or the passing of time from mating. The combined data for all generations showed a significant drop from *a* to *b*, *b* to *c*, and *c* to *e*, but not from *c* to *d*. Examination of the data for the separate series of matings showed that when in *a* vials the percentage of biparentals is higher than the average in *a* vials for all the series in the experiment, a higher percentage is maintained in each of the five vials through which the females are transferred. When the percentage for *a* vials for one series is less than the average, a lower percentage is found throughout all the vials.

Correlation of the Ratios

Attention has been called to the higher percentage of biparentals shown by groups of fraternities having no biparental males as contrasted with groups of fraternities having such males. Since a lower ratio of biparentals was thus found to be associated with the presence of biparental males, it was suspected that comparisons of the ratios within fraternities containing biparental males might show negative correlation. The correlation value was found to be .157 ± .075 for 170 fraternities in the selection experiment, and to be .189 ± .081 for the 141 fraternities of the 11-*o* experiment. Thus in these two experiments a low positive correlation was found to exist between the ratios.

Magnhild M. Torvik, summarizing data collected from experiments conducted by E. J. Wenstrup, found a low negative correlation to exist between the ratios, the value being — .192 ± .087. Wenstrup's experiments were conducted with males from segregating generations after crosses between wild-type stocks Nos. 1 and 11. These males were mated with orange-eyed females of stock No. 3 which was closely related to stock No. 1 but unrelated to stock No. 11. In the selection experiment the material was highly inbred after the initial cross of somewhat related stocks. In the 11-*o* experiment the males were of one stock while the females were grades from an unrelated stock. The significance of the differences is doubtful.

Reciprocal Matings

It has been shown by Anna R. Whiting (1925) and by Whiting and Anderson (1932) that the production of males among biparentals depends on both parents. To determine whether one parent might have more influence than another on the actual ratio, four sets of reciprocal matings were made (Table VII).

Forty-four matings of cantaloup-eyed females by orange-eyed males from stock No. 3 and 49 reciprocal matings showed an unusually low percentage of males among biparentals. The difference between the two crosses was 1.31 ± .50 per cent higher for the orange females by cantaloup males. The percentage of biparentals was higher by 6.3

± 1.7 per cent in the reciprocal. Since the biparental ratio depends so much on the degree of fertilization, even this difference may not indicate any fundamental dissimilarity of the two crosses. Reciprocal matings of cantaloup and ivory-eyed wasps of stock No. 17 gave somewhat similar results. In these crosses biparentals were black-eyed, while azygotic males showed the maternal trait.

A third set of reciprocal matings involved stocks *D-o*, previously described, and reduced (black eyes, reduced wings). *D-o* females by *r* males produced orange azygotic males and black biparentals. Reduced females by *D-o* males gave black reduced azygotic males and black normal-winged biparentals. The percentages were not significantly different for the two crosses.

A fourth set of reciprocal matings involved 11-*o* and reduced. No biparental males were produced although there was some reason to expect them, since 11-*o* came originally from a cross of stocks 11 and 3, to which latter reduced is related.

Table VII

Reciprocal Matings

Matings	Matro-clinous males	Bipar-ental males	Females	Biparental males × 100 / Biparentals	Biparentals × 100 / Total
c ♀♀ × No. 3 ♂♂	1028	3	1299	.23±.13	55.9±1.03
No. 3 ♀♀ × *c* ♂♂	658	10	637	1.54±.48	49.6±1.4
No. 17 ♀♀ × *c* ♂♂	561	8	637	1.24±.43	53.5±1.4
c ♀♀ × No. 17 ♂♂	516	4	718	.55±.27	58.7±1.4
D-o ♀♀ × *r* ♂♂	1091	43	1599	2.6 ±.25	60.1± .9
r ♀♀ × *D-o* ♂♂	546	11	906	1.2 ±.23	62.7±1.2
11-*o* ♀♀ × *r* ♂♂	572		1847		76.4± .8
r ♀♀ × 11-*o* ♂♂	342		600		63.7±1.5

The third and fourth sets of matings serve as evidence, in addition to that reported by others, showing that both parents are necessary for the production of males among biparentals. Reduced males crossed to *D-o* females sired biparental sons but failed to sire them when crossed to 11-*o* females. If the reaction depends on the males both crosses should have given biparental sons. On the other hand, reduced females crossed to 11-*o* males gave no biparental sons but did when crossed to *D-o* males. In this case, if the reaction depends on the female, both crosses should have yielded similar results. Thus it is shown that the reaction depends on both parents, and that whatever mechanism causes the production of these anomolous males occurs at fertilization or soon afterwards.

It is of interest to see that in these reciprocal crosses, where the percentage of males among biparentals is very low, the biparental ratio is always less for the cross having the greater proportion of biparental males.

Fertility of Biparental Males

Anna R. Whiting (1925) and Magnhild M. Torvik (1931) have shown that a large proportion of biparental males are sterile, and that those which are fertile produce very few daughters. E. J. Wenstrup has suggested (unpublished) that biparental males might be more likely to be fertile when they are members of a fraternity having a high ratio of such males. Fifty-seven biparental males, most of which were taken from fraternities having ratios above 25 per cent, were each mated to a virgin female. Each male was tested once, being observed to mate, and then left with the female three or four days. Only four of the males were fertile, and no association between fertility and the ratio of their occurrence was apparent.

Discussion

The most important aspect of the problem of male biparentalism is the composition or genetic constitution of such males. Knowledge of the varying proportions in which they are produced in different crosses contributes to the solution of this problem and supports the theory of sex-determination recently suggested by P. W. Whiting (1933).

According to this theory females have two sets of autosomal chromosomes, $2A$, and two sex chromosomes, X and Y. Azygotic males, developing from unfertilized eggs, are $A + X$ or $A + Y$. Fertilization of an $A + X$ egg nucleus by an $A + Y$ sperm, or the reciprocal, gives rise to a female. In crosses of unrelated stocks no fertilization of $A + Y$ by $A + Y$ or $A + X$ by $A + X$ occurs, but in related stocks such fertilizations may occur, giving rise to biparental males. Haploid males are $A + X$ or $A + Y$, females are $2A + X + Y$, and biparental males are either $2A + 2X$ or $2A + 2Y$.

It may be supposed that crosses of unrelated stocks fail to produce biparental males because of selective fertilization, there being incompatibility between like egg and sperm nuclei. When related stocks are crossed, this incompatibility is reduced.

Data presented in this paper are held to support the view that certain hereditary factors allow the reaction which produces biparental males to take place, and that other factors, of a modifying nature, determine the percentage of such males among the biparentals. The first group of factors may be thought of as allowing fertilization between like gametes to occur infrequently. Modifying factors may either increase the frequency of like fertilizations, or possibly may render young stages more viable which are developing from such fertilizations.

Some mechanism does reduce the number of biparental offspring from matings producing biparental males. Anna R. Whiting (1925) first reported this fact. It will be recalled that for the 11-*o*, 25-*o*, and 31-*o* experiments a lower ratio of biparentals is typical of fraternities including biparental males, as opposed to fraternities lacking them. This also suggests that fertilization by related sperm tends to prevent the development of biparentals.

In 1923 Anna R. Whiting (Whiting and Anderson, 1932) made daily observations on oviposition and development of progeny of 17 females, which were of several kinds. Females, bred as virgins, crossed to unrelated males, or crossed to related males, laid about the same number of eggs. Hatchability of eggs from the last group was significantly less than from the first two. Fertilization by related sperm was considered to have a lethal effect. In the cross of related stocks, biparental males, if produced, were not detectable.

Selection in several directions, in the selection experiment, indicated that the strain was practically homozygous for factors affecting male biparentalism, after the F_{21} generation. However, in the F_{29} one fraternity out of nine did not contain biparental males, in the F_{27} six of nineteen lacked such males, and in the F_{25} there were seven of forty lacking them. In the F_{31} generation all fraternities (17) included biparental males. Examination of the data for the individual fraternities of generations 25, 27, and 29 reveals that practically all fraternities lacking biparental males had fewer biparentals than several other fraternities which included a single biparental male. For example, in the F_{27} the six fraternities having no biparental males had, respectively, 17, 39, 37, 18, 11, and 20 females. Other fraternities having a single biparental male had, respectively, 41, 56, 43, and 32 females. Thus it is probable that the stock was practically uniform genetically and that it was merely a matter of chance that some fraternities had no biparental males.

No exceptions were found to the rule that unrelated stocks do not produce biparental males. The grading of one stock up to another, as was done in the 11-*o*, 25-*o*, and 31-*o* experiments, was accompanied by the presence of biparental males, and a gradual increase in the incidence of their occurrence. Increasing the degree of relationship between parental stocks apparently allowed more fertilizations by like sperms. This decrease in the incompatibility between like gametes is probably due to the attainment of homozygosity for more and more factors affecting male biparentalism. The somewhat different results obtained in the four experiments were probably due to the presence of different factors in the wild-type stocks used.

Summary and Conclusions

1. Selection was effective in increasing the proportion of matings producing biparental males, and in some cases, the proportion of these males in the fraternities including them.
2. Crosses of unrelated stocks do not produce biparental males. When recessive females derived from the F_1 generation are mated to the parental stock having the dominant character, biparental males are produced. Subsequent grading up to this parental stock increases the proportion of such males.
3. Hereditary factors determine the production of biparental males and modify the numbers produced.
4. Fraternities containing biparental males have a lower proportion of biparentals than do fraternities lacking such males.
5. There is apparently no association between fertility of biparental males and the incidence of their occurrence.
6. Different inbred wild-type stocks have different hereditary factors affecting male biparentalism.
7. Results are in full agreement with the theory of sex-determination recently proposed by P. W. Whiting.

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THE CULTIVATION OF A CELLULOSE-DIGESTING FLAGELLATE, TRICHOMONAS TERMOPSIDIS, AND OF CERTAIN OTHER TERMITE PROTOZOA[1]

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Introduction

The cultivation of some of the intestinal wood-feeding flagellates of termites, known to be necessary for the existence of their host on a cellulose diet (Cleveland, 1924), was attempted with three main purposes in mind. These were: (1) to find what inorganic environment was suitable for these highly specialized protozoa; (2) to obtain in culture for the first time a strictly cellulose-feeding protozoön and to study its nutritional requirements; (3) to discover in what way the action of the protozoa on cellulose furnishes food to their insect host.

For most of the work the large Californian termite, *Termopsis angusticollis,* served as the source of material, although the eastern termite, *Reticulitermes flavipes,* and the roach, *Cryptocercus punctulatus,* were also used. The intestinal fauna of *Termopsis angusticollis* consists of seven species of flagellates of which three, *Trichonympha campanula, T. collaris* and *T. sphærica,* are hypermastigotes, while the other four, *Trichomonas termopsidis, Tricercomitus termopsidis, Hexamastix termopsidis,* and *Streblomastix strix* are polymastigotes. All except the last three feed regularly on cellulose.

Culture Experiments

By means of preliminary experiments conducted in culture cell slides, I found that the most favorable osmotic pressure for the protozoa of *T. angusticollis* is that of a 0.3 to 0.4 per cent sodium chloride solution, that the favorable pH range is 6.8 to 7.2, that a ratio of 97 equivalents of Na ion to three of Ca is an optimum one, and that an excess of Na over K is more favorable than the reverse.

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[1] Part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Division of Biology of Harvard University.

[2] It gives me pleasure to acknowledge the helpful interest and advice of Professor L. R. Cleveland, under whom the greater part of this work was done. I am also much indebted to Dr. A. E. Navez and to Professor W. J. Crozier. Thanks are due to Professors T. C. Nelson and J. Allison of Rutgers University, where the work was begun.

Many balanced salt solutions were made conforming more or less to these requirements. The anions used in most of the solutions were chloride, phosphate, bicarbonate, and citrate. Those solutions in which the protozoa were able to survive in good condition for at least a day were then used, with certain nutrients added, for actual culture experiments.

In the setting up of a culture experiment, test tubes provided with a small amount of powdered cellulose and a little Merck's powdered animal charcoal were used. The cellulose was prepared by dissolving Whatman filter paper No. 40 in Cross and Bevans' reagent (1 part by weight $ZnCl_2$, 2 parts concentrated HCl) and reprecipitating in water. The precipitated cellulose was filtered off on a large Buchner funnel, washed with tap water till free of chloride and then with distilled water, and allowed to air-dry. It could then be easily ground up in a mortar to a fine white powder containing many particles small enough to be ingested even by the smallest cellulose-feeding flagellates. The tubes holding the cellulose and charcoal were sterilized either in the autoclave or by dry heat. Each tube then received approximately 8 cc. of the liquid medium, which had been previously sterilized by filtration through a Berkefeld N filter. The liquid in the tubes was covered with sterile vaseline, which effectively prevented any rise in pH due to the escape of CO_2 from the medium.

In most cases, each tube was inoculated with the entire hindgut of a termite. The termite was first disinfected on the outside by a 15 to 40-minute immersion in 1 : 1000 $HgCl_2$ (this did not injure the protozoa) followed by washing in 95 per cent alcohol and in sterile water. The gut was removed with sterile instruments and placed in the culture medium. The liquid was then again covered with sterile vaseline or left exposed, depending on the particular experiment. The tubes, kept at room temperatures in a large closed cupboard, were examined only once or twice a week, since the growth of the protozoa, if any, was always slow.

By these methods a medium (Solution *A*, Table I) was found in which, with the addition of the proper nutrients, three termite flagellates have been cultured for over three years. Two of these, an as yet undescribed *Trichomonas* from *R. flavipes,* and *Tricercomitus termopsidis,*[3] do not require cellulose. The third, *Trichomonas termopsidis,* must have cellulose and small amounts of blood serum. Cultures of this organism were used in several experiments described in the second part of this paper.

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[3] Full details concerning the cultivation of *Tricercomitus termopsidis* and its method of "encystation" are given in a separate paper to appear in *Arch. f. Protistenkunde.*

Of the hypermastigotes, only *Trichonympha sphærica* was able to survive several days in Solution *A* plus Loeffler's dehydrated blood serum (.001 to .2 per cent) with cellulose and charcoal, but no multiplication took place. A series of changes in the salt composition of Solution *A* led to a solution in which (with .001 per cent Loeffler's blood serum, cellulose, and charcoal) *Trichonympha sphærica* lived several weeks and multiplied slightly. This solution was tried with a variety of nutrients (casein, peptone, amino acids, nucleic acid, etc.) and under a variety of physical conditions (as in collodion bags surrounded by large volumes of sterile culture fluid), but no improvement was effected. Further changes in the salt composition finally gave Solution *U* (Table I) in which (with .01 per cent Loeffler's blood serum, cellulose, and charcoal) good initial cultures of *T. sphærica* were obtained. The organisms were usually most abundant two weeks after inoculation of the tube and grew best when the liquid was not covered with vaseline. At the time of maximum population the pH of the cultures was 7.4. After the second week the pH continued to rise, while the number of protozoa decreased slowly, some surviving as long as six weeks. Subcultures, made with 0.5 to 1 cc. of material, showed fair growth in a small percentage of cases, but no second subculture could be effected. Slight changes in the composition of Solution *U* and in its pH, the use of nutrients other than Loeffler's blood serum, and variations in the degree of exposure to air all failed to produce any improvements. Excellent multiplication in many of the initial cultures and in a few of the first subcultures, but never any multiplication in any of the

TABLE I

Salt	Grams per liter distilled water	
	Solution A	Solution U
NaCl	1.169	2.164
$NaHCO_3$	0.840	0.773
$Na_3C_6H_5O_7.2H_2O$ (citrate)	2.943	1.509
$NaH_2PO_4 . H_2O$	0.690	0
KCl	0.745	0
KH_2PO_4	0	1.784
$CaCl_2$	0.111	0.083
$MgSO_4$	0	0.048

second subcultures, suggested the possibility that some essential substance present in the termite's gut is either used up or removed by dilution. Accordingly, a number of termites were defaunated by oxygenation (Cleveland, 1925) and a series of first and second subcultures was then made, with each tube receiving a gut aseptically removed from one of the defaunated termites. Few good first subcultures and no second subcultures were obtained.

It is nevertheless interesting that an organism as highly specialized, both morphologically and physiologically, as *T. sphærica* should have been able to live and multiply to a considerable extent in a relatively simple culture medium. It is also noteworthy that in the case of the three polymastigotes as well as in the case of the hypermastigote, the salt composition of the medium is of the utmost importance. Thus *Trichomonas termopsidis* could be cultured continuously only in Solutions *A* and *U* and not in several other very similar media. Moreover, although Solutions *A* and *U* differ from each other only in the presence of $MgSO_4$ and of more phosphate and less citrate in the latter, yet these small differences were sufficient to make all the difference between mere survival of *Trichonympha sphærica* in *A* and active multiplication in *U*.

TRICHOMONAS TERMOPSIDIS IN CULTURE; ITS NUTRITION AND ITS ACTION ON CELLULOSE

The xylophagous flagellate *T. termopsidis* has been maintained in culture since October, 1930, in Solution *A* with 0.2 per cent Loeffler's blood serum, cellulose, and charcoal. The liquid in each tube is kept covered with a layer of vaseline except for a short time when the vaseline seal is broken to permit examination. The pH of the medium, originally 7.0 to 7.2, never goes below 6.8, regardless of the age of the culture.

Subcultures are made from parent cultures two, three, or four weeks old, using about 0.3 cc. of material to a tube. The inoculum is removed from the very bottom of the culture, for the flagellates are rarely present more than a quarter of an inch above the bottom. Before the use of charcoal was begun, growth failed to appear in some of the subcultures, but ever since then the subcultures have been completely successful. In most subcultures growth is slow during the first week, reaching a value of 0.2 to 1 organism per low-power field (10 such fields counted in a sample of one drop from the bottom of the tube). At the end of the second week from 1 to 5 organisms per field are present, and at the end of the third week from 8 to 20. After the third week the cultures will remain in a stationary state for a week or even several weeks, after which time the organisms begin to die off. There were considerable variations from this usual state of affairs. Some few cultures never got beyond one or two organisms per field, while in others there might be fifty or more.

The *Trichomonas termopsidis* in a culture, up to the time of the fourth or fifth week, are nearly all very active and perfectly normal in appearance, and their bodies contain varying numbers of cellulose particles. Most of the flagellates are from 30 to 50 μ long, although in very young cultures, where division forms are numerous, the smaller organisms predominate. In some old cultures, giant forms and multiple-fission forms have been encountered which can ingest very large pieces of cellulose. All the cultural forms are so obviously like those seen in termites that no detailed morphological study of them has been made. (For the morphology of *T. termopsidis* see Andrews, 1925, and Kirby, 1931.)

Trichomonas termopsidis appears to require very little, if any, oxygen. Not only do the organisms grow best under a vaseline seal, but good growth could also be obtained in tubes connected by means of paraffined stoppers and glass tubing to other tubes containing alkaline pyrogallol, thus creating anærobic conditions. Moreover, exposure of the organisms to atmospheric oxygen is toxic. Thus, when exposed to air in a thin layer of culture fluid in a moist chamber the flagellates rapidly round up and die, while if similarly exposed to nitrogen they continue to swim about actively for several days. A current of air bubbled through a culture at the rate of 60 bubbles per minute killed all the protozoa in less than 24 hours, while a current of nitrogen bubbled through another portion of the same culture at the same rate had no effect.

A bacteriological study of the cultures of *T. termopsidis*, made when the strains were several months old, revealed that only one species of bacteria (derived from the termite's gut) was present. This organism, a short Gram-negative bacillus, grows well at room temperature in broth, as well as in Solution *A* with only $(NH_4)_2SO_4$ as its source of nitrogen. It ferments glucose with gas production, but it does not attack cellulose or cellobiose and cannot live in media ordinarily used for cellulose-fermenting bacteria. Many attempts have been made, using a variety of methods, to free the cultures of this last contaminating organism, but none was successful.

Although the ideal of a bacteria-free culture has thus not been attained, it has nevertheless been possible to perform some experiments concerning the nutrition of *Trichomonas termopsidis*. In one experiment, subcultures were made from healthy and comparable parent cultures growing in the standard medium into media in which the Loeffler's dehydrated blood serum was replaced by 0.1 per cent Difco desiccated blood serum, 0.1 per cent beef broth, 0.1 per cent Bactopeptone, 0.1 per cent cystine plus 0.2 per cent glycine, 0.01 per cent cystine plus 0.01 per cent glycine plus 0.01 per cent tyrosine, 0.1 per cent asparagin or 0.1 per cent Difco hydrolyzed blood serum. Growth occurred only in media containing blood serum or hydrolyzed blood serum. In other series of experiments the cellulose was replaced by rice starch, dextrin, inulin, glycogen, cellobiose, agar, cellulose tri-acetate, or cellobiose octa-acetate. No growth of the flagellates took place, although controls containing cellulose always showed excellent growth. *Trichomonas termopsidis* in culture appears to be specific for cellulose as its carbon source. In this respect it, like most of the other symbiotic flagellates of termites, closely resembles certain bacteria, such as *Spirochæta cytophaga* (Hutchinson and Clayton, 1919) and *Bacillus cellulosæ-dissolvans* (Khouvine, 1923), which are likewise limited to cellulose as their carbon source.

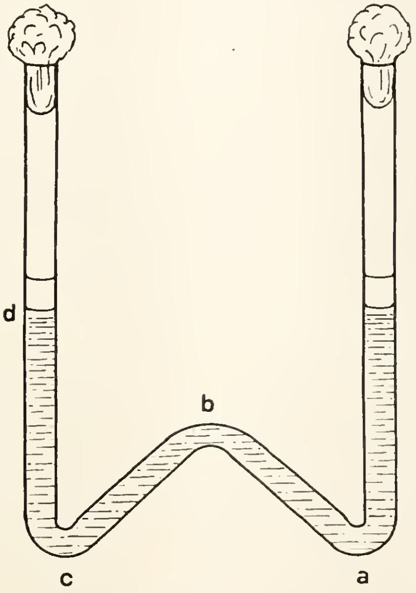

Fig. 1. Diagram of M-tube.

The maintenance of *Trichomonas termopsidis* for several years on a cellulose diet, its inability to live in culture when the cellulose is replaced by other polysaccharides, and the extraction from the cultivated organisms of a cellulase (Trager, 1932) prove that this flagellate is capable of digesting cellulose.

Since the bacillus present in the cultures of *Trichomonas termopsidis* is a very active glucose fermenter, no hope was entertained of being able to demonstrate unequivocally the production of glucose by cultures of the flagellate. Tests with Benedict's solution of the fluid of active cultures were always negative. Some very strong indirect evidence for glucose production was, however, obtained. If an active culture of the trichomonads was divided into two equal parts, of which one was heated to 43° and held there for 10 minutes, thus killing all the protozoa without injuring the bacteria, and if each portion was then placed in a fermentation tube or in a test tube with a heavy layer of vaseline over it, then, after the lapse of several days, gas appeared only in the tube with the live protozoa. To determine whether this gas was produced by the bacteria from glucose formed through the activities of the protozoa, or whether it was produced directly by the protozoa, the following experiment was performed. Twenty active cultures of *T. termopsidis* were mixed in a large sterile tube and centrifuged. The supernatant liquid, determined by microscopic examination to be free of protozoa, was removed completely, and then the residue, containing the protozoa, was resuspended in 5 cc. of this supernatant liquid. Four tubes, shaped like inverted M's (Fig. 1), made from 6 mm. glass tubing and previously plugged with cotton and sterilized, were now set up as follows. Numbers 1 and 2 received each two pipettefuls, and Nos. 3 and 4 three pipettefuls, of the protozoa-free supernatant liquid. The tubes were manipulated so that no air-bubbles remained in them. To one arm, suitably marked, of Nos. 1 and 3 was then added one pipetteful of the protozoan suspension, very carefully and in such a manner that the protozoa settled in notch *a* of the tube and did not rise to the level of notch *b* (Fig. 1). The remainder of the protozoan suspension was then heated to 43° C. and kept at this temperature for 10 minutes. One pipetteful of this dead suspension was then added to one arm of Nos. 2 and 4. The liquid in both arms of all four tubes was covered with a heavy layer of vaseline. In such an arrangement, it is evident that most of the gas formed should accumulate at notch *b* and that, if the protozoa cannot swim up over notch *b*, then any gas appearing in notch *c* or under the vaseline at *d* (Fig. 1) could not possibly have been formed by the protozoa. Within one week after the date of setting up the experiment, in both tubes 1 and 3 gas was present at notch *c* as well as at notches *a* and *b*. Microscopic examination of samples removed from notches *a* and *c* showed that protozoa were present only in notch *a*. In tubes 2 and 4 there was no gas whatever. This proves conclusively that, in tubes 1 and 3, the protozoa present in notch *a* produced by their action on cellulose a soluble substance which diffused over toward notch *c* and was there fermented by the bacteria. That this substance was glucose seems almost certain, in view of the facts that glucose and cellobiose are the only simple sugars formed from cellulose and that the bacillus present readily ferments glucose with gas production, but cannot utilize cellobiose. That this glucose was not produced as the result of the extra-cellular action of cellulase is indicated by the fact that, although cellulase could be demonstrated in the supernatant fluid of old degenerating cultures, it could not be demonstrated in the supernatant fluid of active healthy cultures such as were used for the experiment detailed above. It thus appears that *T. termopsidis* and other symbiotic intestinal flagellates ingest cellulose, digest it to glucose, and then excrete part of the glucose, sharing it with their insect host.

Summary

By means of preliminary experiments, the osmotic pressure, pH, and mono- to bivalent ion ratio most favorable to the survival of the symbiotic intestinal flagellates of termites were determined. On the basis of these and other facts, balanced salt solutions were constructed and tested, with the addition of cellulose and low concentrations of protein, as culture media.

In this way a medium was obtained in which the xylophagous flagellate, *Trichomonas termopsidis,* from *Termopsis angusticollis,* has been cultured for over three years. A *Trichomonas* from *Reticulitermes flavipes,* and *Tricercomitus termopsidis* from *Termopsis angusticollis* were cultured in the same medium. The last two organisms did not require cellulose.

In a somewhat different medium, excellent initial cultures of the hypermastigote, *Trichonympha sphærica,* from *Termopsis angusticollis,* were obtained, and the organisms could be carried through a first subculture but not through a second. Attempts to improve the medium so as to secure continuous cultivation failed.

Experiments with *Trichomonas termopsidis* showed that it cannot utilize any carbon source other than cellulose. This protozoön probably secretes glucose, which is used by its insect host.

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THE SEDIMENTATION CONSTANTS OF THE RESPIRATORY PROTEINS

THE SVEDBERG AND ASTRID HEDENIUS

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The ultracentrifugal technique developed in this laboratory enables us to define the mass and shape properties of a high molecular substance by means of two data, the molecular weight and the sedimentation constant (Svedberg, 1934). The latter one, which is a function of the mass and shape of the molecule, is the easiest to measure with a fairly high degree of precision. The error in the mean value from a few—say ten—absolute determinations is about 1 per cent. Relative measurements can be arranged so as to give an accuracy of around 2 per cent in a single experiment. The amount of substance required for a determination is of the order of 0.001 gram. If the molecules to be studied possess characteristic light absorption in the visible or ultraviolet part of the spectrum, it is possible to make the measurement regardless of the presence of other substances in the solution. In many cases the method further allows us to measure simultaneously the sedimentation constants of the different molecular species in a mixed solution. The determination of the sedimentation constant therefore constitutes an efficient tool for the study of high molecular substances dissolved in native fluids. On the following pages a report will be given of an attempt to characterize the respiratory proteins throughout the animal kingdom by their sedimentation constants (see Svedberg and Hedenius, 1933; Svedberg, 1933).

METHOD

A small quantity of the solution to be studied (0.1 to 1.5 cc.), enclosed in a sector-shaped cell between plane-parallel windows of crystalline quartz, is exposed to the influence of a very strong field of force in a special centrifugal instrument, the ultracentrifuge. In order to avoid convection currents due to temperature disturbances, the rotation takes place in hydrogen of 20 mm. pressure and the surface of the solution is covered with a layer of oil. From time to time photographs of the rotating column of solution are taken so as to record the movement of the boundary between solvent and sedimenting solute. A concentration scale is photographed on the same plate. The pictures are then registered by means of a microphotometer and the curves obtained used for the calculation. If the field of force is strong enough, the rate of settling can be measured and from this the sedimentation constant, defined as the velocity of sedimentation in a field of unit strength reduced to water of 20° as solvent, can be calculated.

If x is the distance from the centre of rotation, t the time, ω the angular velocity, η the viscosity and ρ the density of the solvent, η_0 the viscosity and ρ_0 the density of water of 20°, and V the partial specific volume of the solute, the sedimentation constant is given by the expression:

$$s_{20^{\circ}} = dx/dt \, . \, 1/\omega^2 x \, . \, \eta/\eta_0 \, . \, \frac{1 - V\rho_0}{1 - V\rho}.$$

From previous protein investigations of this laboratory it is known that V varies very little from one protein to the other. The fact that V only figures in a correction term makes it unnecessary to determine it for every protein studied. This circumstance is of considerable importance because of the difficulty of procuring enough material for density determinations in the case of many of the invertebrates. In the following the mean value $V = 0.745$ was used.

The active group of the respiratory pigments confers upon these proteins characteristic absorption bands in the visible and near ultraviolet spectrum. If a suitable wave-length is chosen for the illumination, it is therefore always possible to measure the sedimentation of the molecules of a respiratory pigment undisturbed by the presence of other blood proteins. A sample of blood can be studied in the ultracentrifuge directly after drawing it and eventually diluting it with a salt solution (or laking the blood corpuscles) without subjecting it to any chemical treatment at all. This simplified procedure eliminates serious causes of error in the case of the more unstable pigments.

Four types of respiratory blood proteins are known, red pigments (erythrocruorin, hemoglobin), green pigments (chlorocruorin), blue pigments (hemocyanin) and pigments of reddish-brown color (hemerythrin) (Redfield, 1933). As a rule we have used the mercury lines 577–79 mμ and 546 mμ in combination with a Wratten K3 filter for the study of the red and green proteins, the mercury line 366 mμ with a nickel oxide glass filter for the hemocyanins as well as for mixtures of red or green proteins with hemocyanins and line 546 mμ with a Wratten n:r 77 filter for the hemerythrins. The pictures in the visible spectrum have been taken on Ilford rapid process panchromatic plates while for the ultraviolet we have used Imperial process plates.

For those species the blood of which contains a respiratory protein of high molecular weight at least two determinations have been made, one for the blood in question alone, and one in mixture with the blood of a standard species. This last kind of measurement enables us to compare all the sedimentation determinations with a few well-established constants. The absence of a double boundary in the case of a mixture of two proteins of high molecular weight, and accordingly low diffusion, has been taken as a criterion of the identity of the sedimentation constants (limit of error about ± 2 per cent). On the other hand, the existence of two different sedimentation constants lying close together has always been checked by applying this mixture test.

The blood samples have mostly been diluted down to a pigment concentration of 0.1–0.3 per cent in order to avoid viscosity errors. A few check runs on undiluted blood have been done, however. As a rule we have used 1 per cent NaCl as diluting agent. The natural pH of the blood is often very close to the alkaline stability limit of the respiratory protein contained in it. In such cases, where more than one kind of molecular mass exists at this border, the samples have been diluted with a suitable buffer solution to keep the pH down. The sedimentation constant of the state of aggregation which possesses the widest pH stability range has always been chosen to characterize the protein in question. The sedimentation constants of dissociation or aggregation states observed have been given in parenthesis in the tables.

Most of the invertebrates have their blood pigment dissolved in the plasma. The echinoderms, the lamellibranchians, some of the worms and, of course, all the vertebrates, however, have red blood corpuscles. To bring the pigment into solution the erythrocytes were laked with distilled water and filtered off. Sodium chloride was then added to bring the salt content up to 1 per cent. In cases where we had at our disposal a quantity of blood sufficient for separation of the pigment from colorless proteins eventually present the corpuscles were washed on the centrifuge with sea water or with 1 per cent NaCl solution before laking.

It was found during this investigation that the blood samples obtained from invertebrate animals were often apt to deteriorate rapidly even when kept at 0° C. This was especially pronounced in the case of very small species where it was impossible to avoid contamination of the blood by coelomic fluid or gland excretions. At least one run for each species was therefore carried out immediately after drawing the blood. Tests with samples of frozen blood, however, showed that even the most unstable types could be stored in this state for months without deteriorating. During the latter part of this investigation constant use was made of this circumstance.

TABLE I

Chætopoda: Polychæta, Hirudinea

Pigment dissolved in plasma. Standard species for mixture test, *Nereis virens* (Sars). Solvent, 1 per cent NaCl; thickness of column of solution 1.2 cm.; centrifugal force about 65,000 times gravity.

	Origin of blood	Dilution of blood	Mixed with blood of	$s_{20^\circ} \times 10^{13}$	Mixture test deviation from standard
					per cent
Type of pigment: erythrocruorin	Eunicidæ: Lumbrinereis fragilis (Müll.) Kristineberg, Sweden	10		58.0	–
		20	Nereis virens	59.8†	±3
	Nereidæ: Nereis virens (Sars) Kristineberg, Sweden	10		58.6*	–
		20		61.5*	–
		15		56.6	–
		30		57.6‖	–
		20	Lumbrinereis fragilis	61.5†	–
		20	Arenicola marina	57.0*	–
		15	Eumenia crassa	59.0	–
		20	Pectinaria belgica	54.1	–
		13	Polymnia nebulosa	59.1	–
		20	Sabella pavonia	53.8	–
		20	Hæmopis sanguisuga	57.6	–
		20	Hirudo medicinalis	58.5	–
		20	Lumbricus terrestris	58.4*	–
		10	Planorbis corneus	56.2	–
		20	Helix pomatia	59.6§	–
		20	Sepia officinalis	58.9	–
	Arenicolidæ: Arenicola marina (L.) Kristineberg, Sweden	20	Nereis virens	57.8*	±1
	Scalibregmidæ: Eumenia crassa Oersted Kristineberg, Sweden	10		61.4	–
		10	Nereis virens	57.3	±3

TABLE I—*Continued*

	Origin of blood	Dilution of blood	Mixed with blood of	$s_{20^\circ} \times 10^{13}$	Mixture test deviation from standard
					¶ *per cent*
Type of pigment: erythrocruorin	Amphictenidæ: Pectinaria belgica (Pall.)				
	Kristineberg, Sweden	5		54.6(11.7)	—
		20	Nereis virens	54.3(11.7)	±0
	Terebellidæ: Polymnia nebulosa (Mont.)				
	Kristineberg, Sweden	2		56.4	—
		2	Nereis virens	58.3	±1
Type of pigment: chlorocruorin	Chlorhæmidæ: Brada villosa Rathke				
	Kristineberg, Sweden	—		56.0‡	
		2		56.3‡	
	Serpulidæ:				
	Serpula vermicularis L.				
	Kristineberg, Sweden	4		58.8	
	Sabella pavonia Sav.				
	Kristineberg, Sweden	8		52.7	
		6	Nereis virens	54.4	±1
Type of pigment: erythrocruorin	Hirudinidæ:				
	Hirudo medicinalis L.				
	South Europe	25		58.5	—
		25	Nereis virens	57.5	±1
	Hæmopis sanguisuga L.				
	Upsala, Sweden	35		57.7	—
		35	Nereis virens	56.5	±2
			Mean	57.5	±1.5

* Thickness of column of solution 0.8 cm.

† Thickness of column of solution 1.0 cm.

‡ Thickness of column of solution 0.2 cm.; mercury lines 405–436 mμ; Wratten nr 50 filter, Imperial Process plates.

§ Centrifugal force 39,000 times gravity.

|| Centrifugal force 21,000 times gravity; blood frozen for six months, determined by Mrs. I.-B. Eriksson-Quensel.

The following buffer solutions were used:

pH 5.0. 0.0059 M acetic acid, 0.0141 M Na-acetate, 0.8 per cent NaCl.
pH 6.8. 0.01 M Na_2HPO_4, 0.01 M KH_2PO_4, 0.1 M KCl.
pH 8.0. 0.019 M Na_2HPO_4, 0.001 M KH_2PO_4, 0.1 M KCl.

Table II

Chætopoda: Polychæta

Pigment enclosed in corpuscles. Type of pigment, erythrocruorin; solvent, 1 per cent NaCl; thickness of column of solution, 1.2 cm.; centrifugal force about 180,000 times gravity.

Origin of blood	$s_{20°} \times 10^{13}$
Glyceridæ:	
Glycera Rouxii Aud. and M. E.	
Kristineberg, Sweden	3.56
	3.44
Mean	3.50
Capitellidæ:	
Notomastus latericeus Sars	
Kristineberg, Sweden	2.18
	1.98
Mean	2.08

Table III

Chætopoda: Oligochæta

Pigment dissolved in plasma. Type of pigment, erythrocruorin. Standard species for mixture test, *Lumbricus terrestris* L. Solvent, 1 per cent NaCl; thickness of column of solution 1.2 cm. Centrifugal force about 65,000 times gravity.

Origin of blood	Dilution of blood	Mixed with blood of	$s_{20°} \times 10^{13}$	Mixture test deviation from standard
				per cent
Lumbricidæ:				
Lumbricus terrestris L.				
Upsala, Sweden	20		60.6	—
	20		60.0*	
	20		63.1†	—
	20	Nereis virens	63.0‡	—
	20	Tonicella marmorea	62.1	—
	40	Sepia officinalis	59.9	—
	30	Eisenia fœtida	65.6	—
	20	Arion ater	57.7	—
Eisenia fœtida Sav.				
Upsala, Sweden	20		63.5	
	30	Lumbricus terrestris	63.4	±4
		Mean	61.9	

* Blood frozen for 16 days.
† Blood frozen for 49 days.
‡ Thickness of column of solution, 0.8 cm.

In the tables they will be denoted by their pH. The additions of neutral salt have been made in order to repress the disturbances of the sedimentation caused by the electric charge of the protein molecules (Donnan effect).

SEDIMENTATION CONSTANTS

Scolecida

Among the lower worms one representative of the nemerteans was examined, but it was found impossible to draw enough blood from it for a determination of the sedimentation constant.

Annelida

A considerable number of species belonging to this group have been studied. In most cases the respiratory pigment is in solution and sufficient quantities of blood may easily be drawn. A large worm like *Arenicola* may yield up to 0.3 cc., while it requires about a dozen individuals of a small species like *Serpula* to make up 0.1 cc. *Nephthys ciliata, Panthalis oerstedi, Thelepus cincinnatus* and *Terebellides stroemi* were tried without success. Worms with red blood corpuscles are scarce and it is not always easy to extract the pigment without injuring it. With *Echiurus pallasi* and *Priapulus caudatus* we were unsuccessful. Erythrocruorin, chlorocruorin, and hemerythrin but not hemocyanin have been found within this group of animals.

Tables I–III contain the determinations of sedimentation constants.

By adopting the mixture test we were able to prove that the dissolved erythrocruorin and chlorocruorin of all the Polychæta and Hirudinea species have the same sedimentation constant with a mean value of 57.5×10^{-13}. The Oligochæta have a slightly higher constant, viz., 61.9×10^{-13}. Two different and very low constants, 3.50 and 2.08×10^{-13}, were found for the erythrocruorin contained in the corpuscles of the glyceride and capitellide worms. The hemerythrin from the corpuscles of the sipunculoideans has also a low constant, but owing to the scarcity of the material at our disposal (*Phascolosoma vulgare*), accurate values for the sedimentation constant could not be obtained. We have therefore refrained from including these data in the tables. The erythrocruorin of *Pectinaria* is very unstable and decomposes easily, giving rise to a product of sedimentation constant 11.7×10^{-13}. A dissociation product of the same constant has been observed at the alkaline border of the stability range in the case of *Arenicola* erythrocruorin (Svedberg and Eriksson-Quensel, 1933). Sedimentation equilibrium measurements indicate that the appearance of this product represents the breaking up of the molecule into 16 equal parts of weight 207,000.

TABLE IV

Crustacea: Phyllopoda, Malacostraca

Pigment dissolved in plasma. Standard species for mixture test, *Pandalus borealis* Krøyer. Solvent, 1 per cent NaCl or buffer pH 5. Thickness of column of solution 1.2 cm.; centrifugal force, 90,000–165,000 times gravity.

Origin of blood		Dilution of blood	Mixed with blood of	$s_{20°} \times 10^{13}$	Mixture test deviation from standard
					per cent
Type of pigment: erythrocruorin	Daphnidæ: Daphnia pulex de Geer Upsala, Sweden			16.5* †	
				17.2*	
				17.1*	
			Palinurus vulgaris	16.8*	
	Bopyridæ: Athelges sp. Kristineberg, Sweden	7	Planorbis corneus	19.3	
Type of pigment: hemocyanin	Carididæ: Pandalus borealis Krøyer Kristineberg, Sweden	5		16.6	
		5	Palinurus vulgaris	16.8	
		5	Pagurus striatus	18.1	
		5	Homarus vulgaris	17.2	
	Palæmon fabrici Rathke	15	Homarus vulgaris	15.6	

TABLE IV—*Continued*

	Origin of blood	Dilution of blood	Mixed with blood of	$s_{20^\circ} \times 10^{13}$	Mixture test deviation from standard
					per cent
Type of pigment: hemocyanin	Palinuridæ: Palinurus vulgaris Latr.				
	Naples, Italy	8		17.2	
		9		15.3	
		8	Pandalus borealis	15.8	±6
		10	Daphnia pulex	18.1	
	Paguridæ:				
	Pagurus striatus Latr.				
	Naples, Italy	7		16.0‡	
		10	Pandalus borealis	16.7‡	±8
	Eupagurus bernhardus (L.)				
	Kristineberg, Sweden	5	Homarus vulgaris	17.1‡	
		5		16.2‡(21.6)	
		5		17.4‡(22.5)	
			Mean	16.9 (22.1)	

* Thickness of column of solution 0.8 cm.

† Centrifugal force 45,000 times gravity.

‡ Solvent buffer pH 5.

TABLE V

Crustacea: Malacostraca

Pigment dissolved in plasma. Type of pigment, hemocyanin. Standard species for mixture test, *Homarus vulgaris* M. E. Solvent, 1 per cent NaCl or buffer pH 5. Thickness of column of solution 1.2 cm. Centrifugal force 67,000–150,000 times gravity.

Origin of blood	Dilution of blood	Mixed with blood of	$s_{20°} \times 10^{13}$	Mixture test deviation from standard
				per cent
Squillidæ:				
Squilla mantis Latr. Naples, Italy......	10		23.7†	
	10	Homarus vulgaris	23.4†	±3
Nephropsidæ:				
Nephrops norvegicus (L.) Kristineberg, Sweden..........	8		24.4	
	8	Homarus vulgaris	24.7	±1
Homarus vulgaris M. E. Havstenssund, Sweden..........	5		24.6*	
	10		24.3†	
	10	Pandalus borealis	24.2	
	10	Palæmon fabrici	21.7	
	10	Eupagurus bernhardus	22.1†	
	6	Cancer pagurus	22.4	
	10	Carcinus mænas	22.3†	
	6	Astacus fluviatilis	23.7	
	10	Hyas araneus	22.3†	
	8	Nephrops norvegicus	24.4	
	10	Squilla mantis	22.7†	
	10	Calocaris macandreæ	22.6	
	7	Planorbis corneus	23.9	
	10	Planorbis corneus	25.3	
	10	Planorbis corneus	24.8	
	10	Sepia officinalis	23.0	
	12	Octopus vulgaris	22.0	
	5	Chiridothea entomon	22.5	
Astacus fluviatilis L. Dådran, Sweden...	5		23.8	
	10		25.0	
	10		24.1‡	
	6	Homarus vulgaris	23.4	±1

Table V—*Continued*

Origin of blood	Dilution of blood	Mixed with blood of	$s_{20°} \times 10^{13}$	Mixture test deviation from standard
				per cent
Majidæ:				
Hyas araneus (L.) Kristineberg, Sweden	6		24.2†	
	5	Homarus vulgaris	22.2†	
Maja squinado Latr. Naples, Italy	1		26.7	
Cancridæ:				
Cancer pagurus L. Havstenssund, Sweden	5		23.0(15.7)	—
	5	Homarus vulgaris	22.5†	±0
Carcinus maenas (L.) Kristineberg, Sweden	6		24.1†(16.4)	
	5	Homarus vulgaris	22.2†	±0
Idotheidæ:				
Chiridothea entomon L. Härnösand, Sweden	10		23.0(16.1)	
	4	Homarus vulgaris	22.7†(16.0)	±1
		Mean	23.5(16.1)	±1

* Thickness of column of solution 1.0 cm.
† Solvent buffer pH 5.
‡ Thickness of column of solution 0.8 cm.

Arthropoda

Among the numerous species of this group possessing respiratory blood proteins there are several large forms, such as the lobster and the horseshoe crab, which yield large quantities of blood. In the case of *Balanus* and *Branchipus* we were unable to collect enough respiratory pigment for a determination of the sedimentation constant. It is doubtful whether the former has any respiratory pigment at all. Species with blood corpuscles are not to be found among the Arthropoda, all species belonging to this group having their pigment dissolved in the blood plasma. Hemocyanin is the commonest pigment; erythrocruorin is also represented; but chlorocruorin and hemerythrin do not occur here. Tables IV–VII contain the determinations of sedimentation constants.

As shown by the mixture test, five different sedimentation constants were found within this group, viz., 17, 24, 34 × 10^{-13} for the crustaceans; 17, 34, 57 × 10^{-13} for the xiphosurans; 34 × 10^{-13} for the scorpionideans, and the very low constant 2.0 × 10^{-13} for the insects (*Chironomus* larvæ).

The crustacean hemocyanin of constant 23.5 × 10^{-13} gives a dissociation product of constant 16.1 × 10^{-13} at the alkaline border of the stability range. The natural pH of the blood is such that this component is almost always observed in the fresh blood. With time the pH decreases spontaneously and the dissociation goes back reversibly. The appearance of this product probably means the breaking up of the molecule into two equal parts. As shown by Table IV, the molecule of constant 16.9 is the normal one in a number of crustacean species both in the case of hemocyanin and erythrocruorin.

Table VI

Crustacea: Malacostraca;
Arachnomorpha: Xiphosura, Scorpionidea

Pigment dissolved in plasma. Type of pigment, hemocyanin. Solvent, 1 per cent NaCl; centrifugal force about 115,000 times gravity. Thickness of column of solution 1.2 cm.

Origin of blood	Dilution of blood	Mixed with blood of	$s_{20^\circ} \times 10^{13}$	Mixture test deviation from standard
				per cent
Thalassinidæ:				
Calocaris macandreæ Bell Kristineberg, Sweden	4		34.0	
	4	Homarus vulgaris	33.9	
	4	Planorbis corneus	33.7	±4
Limulidæ:				
Limulus polyphemus (L.) Woods Hole, Mass.	5		34.1(61.2)(16.7)	
	4		33.6*(56.9)(16.3)	
Chactidæ:				
Euscorpius carpaticus (L.) Naples, Italy	11		36.6	
	11	Planorbis corneus	31.9	±5
		Mean	34.0	

* Solvent buffer pH 5.

Table VII

Eutracheata: Diptera

Pigment dissolved in plasma. Type of pigment, erythrocruorin. Solvent, 1 per cent NaCl. Centrifugal force about 216,000 times gravity. Thickness of column of solution 0.8 cm.

Origin of blood	Dilution of blood	Mixed with blood of	$s_{20°} \times 10^{13}$	Mixture test deviation from standard
Chironomidæ:				
Chironomus plumosus (larvæ) L.				
Storvik, Sweden	20		1.8	
	20		2.1	
Chironomus sp.				
Upsala, Sweden	12		2.0	
	12		1.9	
	8		2.1	
	8		1.8*	
		Mean	2.0	

* Solvent buffer pH 8.

Limulus presents the unique example of a species the blood of which contains hemocyanin of three different sedimentation constants not only at the alkaline border but also near the isoelectric point. A detailed investigation of the *Limulus* blood by Mrs. I.-B. Eriksson-Quensel has shown that these components exist throughout the whole stability

Table VIII

Amphineura: Placophora

Pigment dissolved in plasma. Type of pigment, hemocyanin. Solvent, 1 per cent NaCl. Thickness of column of solution 1.2 cm. Centrifugal force about 65,000 times gravity.

Origin of blood	Dilution of blood	Mixed with blood of	$s_{20°} \times 10^{13}$	Mixture test deviation from standard
				per cent
Chitonidæ:				
Tonicella marmorea Fabr.				
Kristineberg, Sweden	2.0		60.4	
	2.5		62.8*	
	2.5	Lumbricus terrestris	59.4	±5
		Mean	60.9	

* Centrifugal force 32,000 times gravity.

range. At the acid border the two highest components disappear, giving rise to a new component of constant 23×10^{-13}. According to determinations by K. O. Pedersen, all the components have the same isoelectric point.

Mollusca

From some of the big forms of this group it is easy to draw large amounts of blood in a very pure state. Thus a single individual of the tropical snail *Achatina fulva* yields 50 cc. of blood and a medium-sized *Octopus* or *Eledone* gives as much. On the other hand, there are small forms from which we were unable to get pure blood. This was the case with *Dentalium entale*. Other, somewhat larger, forms such as *Æolis*

TABLE IX

Conchifera: Gastropoda

Pigment dissolved in plasma. Type of pigment, erythrocruorin. Solvent, 1 per cent NaCl; thickness of column of solution 1.2 cm. Standard species for mixture test, *Planorbis corneus* (L.); centrifugal force 65,000–115,000 times gravity.

Origin of blood	Dilution of blood	Mixed with blood of	$s_{20°} \times 10^{13}$	Mixture test deviation from standard
Limnæidæ:				
Planorbis corneus (L.)				
Upsala, Sweden	10		35.1* †	
	10		31.4*	
	15		33.8	
	10	Nereis virens	33.6	
	10	Athelges sp.	34.0	
	12	Homarus vulgaris	32.2	
	15	Homarus vulgaris	34.2	
	15	Homarus vulgaris	32.4	
	15	Calocaris macandreæ	32.2	
	15	Euscorpius carpathicus	30.5	
	12	Helix pomatia	33.7†	
	15	Helix pomatia	35.7†	
	15	Octopus vulgaris	33.9	
	20	Planorbis umbilicatus	32.2	
Planorbis umbilicatus (Müll.)				
Upsala, Sweden	10		36.7*	
	10		36.9*	
	10		37.2*	
	10		32.9*	
		Mean	33.8	

* Thickness of column of solution 0.8 cm.
† Centrifugal force about 41,000 times gravity.

TABLE X

Conchifera: Gastropoda

Pigment dissolved in plasma. Type of pigment, hemocyanin. Solvent 1 per cent NaCl; thickness of column of solution 1.2 cm. Standard species for mixture test, *Helix pomatia* L.; centrifugal force 22,000–60,000 times gravity.

Origin of blood	Dilution of blood	Mixed with blood of	$s_{20°} \times 10^{13}$	Mixture test deviation from standard
				per cent
Paludinidæ:				
Paludina vivipara (L.)				
Upsala, Sweden	10		93.0	
	20		99.2*	
	undil.		97.4‡	
Paludina contecta, Millet				
Germany	4		99.6†	
	4	Helix pomatia	100.3	±2
Littorinidæ:				
Littorina littorea (L.)				
Kristineberg, Sweden	10		99.1*(137.0)	
	2		97.3*(126.8)	
	4	Helix pomatia	96.3	±1
Fasciolaridæ:				
Neptunea antiqua (L.)				
Kristineberg, Sweden	15		107.3†	
	20	Helix pomatia	107.7†	±8
Buccinidæ:				
Buccinum undatum L.				
Kristineberg, Sweden	8		101.4*(131.6)	
	4	Helix pomatia	101.9	±1
Turbinellidæ:				
Busycon canaliculatum (L.)				
Woods Hole, Mass.	20		101.3(138.8)	
	20	Helix pomatia	96.9	±0

TABLE X—*Continued*

Origin of blood	Dilution of blood	Mixed with blood of	$s_{20°} \times 10^{13}$	Mixture test deviation from standard
				per cent
Limnæidæ:				
Limnæa stagnalis (L.)				
Upsala, Sweden	undil.		94.4	
	"		104.1 (60.2)	
	"		96.8	
	"	Helix pomatia	96.6	±3
Pupillidæ:				
Achatina fulva Brug.				
Africa	10		95.7 (63.1, 15.1)	
	10	Helix pomatia	107.4 (64.3, 17.3)	±1
Helicidæ:				
Helix pomatia L.				
Upsala, Sweden	12	Paludina contecta	102.3	
	9	Littorina littorea	95.4	
	12	Neptunea antiqua	99.5	
	9	Buccinum undatum	100.8	
	20	Busycon canaliculatum	96.6	
	15	Limnæa stagnalis	99.2	
	9	Achatina fulva	105.7	
	12	Helix arbustorum	98.0†	
	12	Helix nemoralis	101.3†	
	10	Helix hortensis	101.3*	
	12	Limax maximus	110.0	
	10	Arion ater	99.9†	
	12	Nereis virens	102.7	
	12	Planorbis corneus	99.4	
	10	Planorbis corneus	103.2	
	12	Sepia officinalis	103.2	
	12	Octopus vulgaris	99.5	
	15	Octopus vulgaris	104.1	
	10	Octopus vulgaris	101.4	

Origin of blood	Dilution of blood	Mixed with blood of	$s_{20°} \times 10^{13}$	Mixture test deviation from standard
				per cent
Helix arbustorum L.				
Upsala, Sweden	5		95.3*	
	5		94.3*	
	12	Helix pomatia	96.3†	±2
Helix nemoralis (Müll.)				
Upsala, Sweden	8		101.7*	
	7		100.2*	
	6		99.5*	
	6		95.2	
	12	Helix pomatia	100.8†	±0
Helix hortensis (Müll.)				
Upsala, Sweden	10		99.2*	
	10		98.5*	
	10	Helix pomatia	101.1*	±0
Limacidæ:				
Agriolimax agrestis (L.)				
Upsala, Sweden	10		95.9*	
	10		104.2	
Limax maximus L.				
Länna, Sweden	12		97.0†	
	12	Helix pomatia	111.8†	±2
Arion ater L.				
Länna, Sweden	10		93.7†(59.3)	
	9		110.5(63.2)	
	9		91.4(60.1)	
	10	Helix pomatia	99.9(61.1)‡	
	10	Lumbricus terrestris	(59.2)‡	±3
Arion subfuscus Drap.				
Länna, Sweden	5		(64.3)	
		Mean	100.1 (16.2) (61.9) (133.6)	±1.9

* Thickness of column of solution 0.8 cm.

† Solvent pH 5.

‡ Visible light, K3-filter, maximum of light absorption in the visible spectrum, 600 mμ.

papillosa, Dendronotus arborescens and *Patella vulgata* gave enough blood but did not show any of the absorption bands characteristic of the respiratory proteins.

According to some general remarks in the text-books of physiology (Winterstein, 1925), the blood of the lamellibranchians should, as a rule, contain hemocyanin. We have examined the following species, some of which are fairly large and easy to bleed, viz., *Unio pictorum, Anodonta cygnea, Dreissensia polymorpha, Cyprina islandica, Lævicardium norvegicum, Dosinia exoleta, Tellina crassa, Solen ensis, Solen siliqua, Mya arenaria, Ostrea edulis, Mytilus edulis, Volsella modiolus, Pecten opercularis.* In none of them were we able to detect the presence of any respiratory protein. The blood of the mussels has often a blueish opalescence accompanied by a slight light absorption which might have misled the earlier observers, but it is caused by the presence of coarse suspended particles of unequal size settling rapidly when run in the ultracentrifuge and not by a blood pigment. A few lamellibranchians possess red corpuscles. We have had the opportunity to study only one species of this type, the blood-clam *Arca pexata.* All the other Mollusca have their respiratory protein dissolved in the blood plasma. Hemocyanin is the most common pigment but erythrocruorin occurs in a few species. Chlorocruorin and hemerythrin have not been found in this group. Tables VIII–XIII contain the determinations of sedimentation constants.

TABLE XI

Conchifera: Lamellibranchiata

Pigment enclosed in corpuscles. Type of pigment, erythrocruorin. Solvent, 1 per cent NaCl; centrifugal force about 260,000 times gravity.

Origin of blood	Dilution of blood	Thickness of column of solution	$s_{20°} \times 10^{13}$
		cm.	
Arcidæ: Arca pexata Say Woods Hole, Mass.	5	0.2	3.40
	6	0.4	3.39
	6	0.4	4.09
	6	0.6	3.24*
	20	1.2	3.20
		Mean	3.46

* Solvent buffer pH 6.8; determined by Mrs. I.-B. Eriksson-Quensel.

Table XII

Conchifera: Cephalopoda: Decapoda

Pigment dissolved in plasma. Type of pigment, hemocyanin. Solvent, 1 per cent NaCl. Thickness of column of solution 1.2 cm. Standard species for mixture test, *Sepia officinalis* L. Centrifugal force about 75,000 times gravity.

Origin of blood	Dilution of blood	Mixed with blood of	$s_{20°} \times 10^{13}$	Mixture test deviation from standard
				per cent
Loliginidæ:				
Loligo vulgaris Lam.				
Naples, Italy........	10		56.4	
	10	Sepia officinalis	55.1	±1
	15	Sepia officinalis	55.6	±1
Sepiolidæ:				
Sepiola oweniana d'Orb.				
Kristineberg, Sweden	12		56.3	
	12		56.4	
	20	Sepia officinalis	55.4‡	±0
Rossia oweni Ball				
Kristineberg, Sweden	10		54.6	
	15		57.4	
	15	Sepia officinalis	55.7‡	±1
	12	Octopus vulgaris	55.1†	
Sepiidæ:				
Sepia officinalis L.				
Naples, Italy........	20		54.8*	
	25		57.1	
	20		55.9§	
	20		56.5	
	20		55.2†	
	20	Loligo vulgaris	55.4	
	20	Loligo vulgaris	55.0	
	20	Sepiola oweniana	55.2‡	
	20	Rossia oweni	55.4‡	
	20	Nereis virens	60.4‖	
	20	Lumbricus terrestris	58.4	
	20	Homarus vulgaris	56.1	
	20	Helix pomatia	60.1‖	
	20	Octopus vulgaris	55.6	
		Mean	56.2	±0.8

* Solvent pH 5.0.

† Solvent pH 6.8.

‡ Solvent pH 8.0.

§ Centrifugal force 115,000 times gravity.

‖ Centrifugal force 45,000 times gravity.

TABLE XIII

Conchifera: Cephalopoda: Octopoda

Pigment dissolved in plasma. Type of pigment, hemocyanin. Solvent, 1 per cent NaCl; thickness of column of solution 1.2 cm. Standard species for mixture test, *Octopus vulgaris* Lam. Centrifugal force 65,000 times gravity.

Origin of blood	Dilution of blood	Mixed with blood of	$s_{20°} \times 10^{13}$	Mixture test deviation from standard
Octopodidæ:				*per cent*
Octopus vulgaris Lam.				
Naples, Italy	20		50.6	
	20		49.5	
	15		48.4	
	15	Rossia oweni	48.7*	
	20	Sepia officinalis	49.7	
	20	Eledone cirrosa	50.0	
	17	Homarus vulgaris	50.5	
	15	Planorbis corneus	51.0	
	20	Helix pomatia	53.4†	
	15	Helix pomatia	54.5†	
Eledone moschata (Lam.)				
Naples, Italy	10		48.9*	
Eledone cirrosa (Lam.)				
Kristineberg, Sweden . .	20		46.4	
	20	Octopus vulgaris	49.8	±0
		Mean	50.1	

* Solvent buffer pH 6.8.

† Centrifugal force 35,000 times gravity.

Six different sedimentation constants were found. The hemocyanin of the gastropods has the value 100.1×10^{-13}; the erythrocruorin of the same class 33.8×10^{-13}; the hemocyanin of the Amphineura, the Decapoda and the Octopoda 60.9, 56.2 and 50.1×10^{-13} respectively, and the erythrocruorin of the lamellibranchians 3.46×10^{-13}. The constants 133.6, 61.9 and 16.2×10^{-13} were observed for association and dissociation states of the gastropod hemocyanin. At the natural pH of the blood of the gastropods the dissociation product of constant 61.9, which is probably one-half the normal molecule, almost always appears. It is sometimes accompanied by the component of constant 16.2 (one-eighth of the normal molecule) and very seldom by the association product of constant 133.6. This latter molecule seems to be limited to the marine gastropods. It has been observed in the blood of *Littorina, Buccinum* and *Busycon*.

TABLE XIV

Eleutherozoa: Holothurioidea

Pigment enclosed in corpuscles. Type of pigment, erythrocruorin. Solvent, 1 per cent NaCl; thickness of column of solution 0.4 cm. Centrifugal force 250,000 times gravity.

Origin of blood	Dilution of blood	$s_{20°} \times 10^{13}$
Cucumariidæ:		
Thyone briareus Les.		
Woods Hole, Mass.	6	2.60
	6	2.10
	6	2.95
	9	2.64
		Mean 2.57

Echinoderma

Only one representative of this group possessing respiratory blood pigment has been studied, viz., the sea cucumber, *Thyone briareus.* The protein, which is of the erythrocruorin type, is contained in blood corpuscles. The sedimentation constant is 2.57×10^{-13}. Table XIV gives the determinations.

Tunicata

No respiratory protein is recorded from these animals. We have examined the blood of *Ciona intestinalis* in the ultracentrifuge. It did not show any light absorption due to respiratory proteins.

TABLE XV

Cyclostomata: Hyperoartia, Hyperotreta

Pigment enclosed in corpuscles. Type of pigment, erythrocruorin. Solvent, 1 per cent NaCl + CO. Centrifugal force 225,000–305,000 times gravity.

Origin of blood	Dilution of blood	Thickness of column of solution	$s_{20°} \times 10^{13}$
		cm.	
Petromyzontidæ:			
Lampetra fluviatilis (L.)			
Älvkarleby, Sweden	9	0.4	1.98
	7	0.3	2.09
Myxinidæ:			
Myxine glutinosa L.			
Kristineberg, Sweden	6	0.2	2.33
	6	0.4	2.20
			Mean 2.15

Table XVI

Pisces: Selachii, Dipnoi, Teleostei

Pigment enclosed in corpuscles. Type of pigment, hemoglobin. Solvent, 1 per cent NaCl + CO. Centrifugal force 225,000–285,000 times gravity.

Origin of blood	Dilution of blood	Thickness of column of solution	$s_{20°} \times 10^{13}$
		cm.	
Rajidæ:			
Raja clavata L.			
Kristineberg, Sweden	—	0.6	4.21
	—	0.4	4.34
Lepidosirenidæ:			
Protopterus annectens Owen			
Senegal	2	0.3	4.52
	4	1.2	4.15
Salmonidæ:			
Salmo irideus Gibb.			
Harbo fish-culture, Sweden	4	0.4	4.14
	4	0.4	4.11
Cyprinidæ:			
Cyprinus carassius			
Åkerlänna, Sweden	5	0.4	4.38
	10	1.2	4.25
	6	0.3	4.63*
Anguillidæ:			
Anguilla anguilla (L.)			
Älvkarleby, Sweden	10	0.4	4.21
	10	0.4	4.04
Esocidæ:			
Esox lucius L.			
Upsala, Sweden	—	1.2	4.40
	—	1.2	4.05
Gasterosteidæ:			
Gasterosteus pungitius L.			
Upsala, Sweden	10	1.2	4.45
Percidæ:			
Lucioperca sandra Cuv.			
Upsala, Sweden	—	1.2	4.48
Tautoga onitis (L.)			
Woods Hole, Mass.	10	0.4	4.12
	10	0.4	4.27

Table XVI—*Continued*

Origin of blood	Dilution of blood	Thickness of column of solution	$s_{20^\circ} \times 10^{13}$
		cm.	
Pleuronectidæ:			
Pleuronectes platessa L.			
Kristineberg, Sweden	—	0.4	4.26
Triglidæ:			
Prionotus carolinus (L.)			
Woods Hole, Mass.	10	0.4	4.24
	10	0.4	4.49
Blennidæ:			
Opsanus tau (L.)			
Woods Hole, Mass.	10	0.4	4.21
	10	0.4	4.46
			Mean 4.29

* Frozen for 41 days.

Acrania

A representative of this group, viz., *Branchiostoma lanceolatum*, was tried. We did not find it possible to draw enough blood from it for an ultracentrifugal test.

Vertebrata

The above measurements show that nowhere among the invertebrates has the respiratory protein hemoglobin characterized by the sedimentation constant 4.4×10^{-13} and the active hemin group been met with. In view of this fact it became of great importance to study the red blood pigment of the various classes of the vertebrates in order to find out whether hemoglobin is the only respiratory blood protein of the higher animals. Tables XV–XX contain the determinations of the sedimentation constants.

The blood pigment from the lowest class of the vertebrates, the Cyclostomata, has a sedimentation constant of 2.15×10^{-13} or less than half that of hemoglobin. Sedimentation equilibrium measurement by Mrs. I.-B. Eriksson-Quensel indicates that this pigment which we have classed among the erythrocruorins consists of a mixture of molecules of weight 17,500 and 34,500.

The mean values of the constants for Mammalia, Aves, and Pisces, viz., 4.29, 4.31, and 4.29×10^{-13}, are identical within the limits of error. The pigments from the reptiles and the amphibians very often show a second component of constant 7.14×10^{-13} probably corresponding to a double molecule. This association product seems to increase

TABLE XVII

Amphibia: Urodela, Anura

Pigment enclosed in corpuscles. Type of pigment, hemoglobin. Solvent, 1 per cent NaCl + CO; centrifugal force 195,000–290,000 times gravity.

Origin of blood	Dilution of blood	Thickness of column of solution	$s_{20^\circ} \times 10^{13}$
		cm.	
Salamandridæ:			
Salamandra maculosa Laur.			
Germany	—	1.2	4.89(7.66)
	—	1.2	4.96(6.24)
	10	0.6	4.60*
	—	0.6	4.83*
Bufonidæ:			
Bufo viridis Laur.			
Germany	—	1.2	4.72(7.54)
	—	1.2	4.83(7.27)
Bufo valliceps Wiegm.			
Honduras	—	0.6	4.82†
	—	0.6	4.73*(7.73, 12.53)
Ranidæ:			
Rana temporaria L.			
Upsala, Sweden	—	1.2	4.28
	—	1.2	4.62
			4.73(7.29) Mean

* Centrifugal force 365,000 times gravity.

† Second computation incalculable.

on standing. Sometimes a third component about 12×10^{-13} (probably a triple molecule) was observed in old samples of reptile blood. The constant of the normal component is 4.71×10^{-13} or slightly higher than that for the mammals, birds, and fishes.

The difference is believed to be real. Tests carried out at high speed (72,000 r.p.m. corresponding to a centrifugal force 380,000 times gravity) have not given any indication of a composite character of the 4.7 component. Sedimentation equilibrium measurements will be required to decide whether this deviation is due to a real difference in molecular weight or merely to a slightly different shape of the hemoglobin molecule of the reptiles and amphibians. Some recent determinations of sedimentation constants and molecular weights carried out by K. O. Pedersen on horse blood hemoglobin have shown that this molecule tends to dissociation at high dilutions. We have observed such a phenomenon even more strikingly in one case of cat's blood hemoglobin. Here dissociation into half molecules took place even at moderate dilutions.

TABLE XVIII

Reptilia: Testudinata, Squamata

Pigment enclosed in corpuscles. Type of pigment, hemoglobin. Solvent, 1 per cent NaCl + CO; centrifugal force 220,000–290,000 times gravity.

Origin of blood	Dilution of blood	Thickness of column of solution	$s_{20°} \times 10^{13}$
		cm.	
Testudinidæ:			
Chrysemys picta (Schneid.)			
N. America	—	0.6	4.47(6.97)
	—	0.6	4.38(6.67)
Anguidæ:			
Anguis fragilis L.			
Germany	—	0.3	4.96
	30	0.6	4.47*
	30	0.6	5.00*
Lacertidæ:			
Lacerta vivipara Jacq.			
Germany	—	0.6	4.20(6.96)
	—	0.6	4.90(7.07)
	10	0.6	4.67(7.30)
	20	0.6	4.50†
Chamæleontidæ:			
Chamæleon chamæleon (L.)			
S. Europe	—	0.4	4.57
Colubridæ:			
Coluber longissimus (Laur.)			
S. Europe	5	0.3	4.67
	5	0.3	4.75
	10	0.6	5.04
	20	0.6	4.72
	20	0.6	4.80
	20	0.6	4.66*
	60	2.2	4.98
			Mean 4.69(6.99)

* Centrifugal force 380,000 times gravity.
† Second computation not calculable.

SEDIMENTATION RATIOS

In order to check the above determinations and bind together the higher constants into a system of relative values, a number of ultracentrifugal runs were carried out on mixtures containing two blood pigments of different sedimentation constants. Such relative measurements are possible only in the case of rapid sedimentation where the

TABLE XIX

Aves: Gallinacei, Columbæ, Lamellirostres, Striges, Pici, Passeres

Pigment enclosed in corpuscles. Type of pigment, hemoglobin. Solvent, 1 per cent NaCl + CO. Centrifugal force, 230,000–380,000 times gravity.

Origin of blood	Dilution of blood	Thickness of column of solution	$s_{20°} \times 10^{13}$
		cm.	
Phasianidæ:			
Gallus gallus L.			
Upsala, Sweden	30	0.3	4.13
	30	0.3	4.31
Columbidæ:			
Columba livia L. v. domestica			
Upsala, Sweden	—	1.2	4.46
	—	1.2	4.33
Anatidæ:			
Anas platyrhyncha L. v. domestica			
Upsala, Sweden	40	0.6	4.41
	40	1.2	4.34
Strigidæ:			
Syrnium aluco L.			
Upsala, Sweden	—	0.6	4.28
	—	0.6	4.39
Picidæ:			
Picus viridis L.			
Upsala, Sweden	40	0.4	4.37
	80	1.2	4.23
Paridæ:			
Parus major L.			
Upsala, Sweden	30	1.2	4.35
	15	0.6	4.21
Corvidæ:			
Corvus cornix L.			
Upsala, Sweden	20	0.6	4.45
	44	1.2	4.25
			Mean 4.32

diffusion is low and the boundaries therefore fairly sharp. This condition is not fulfilled for the pigments contained in corpuscles. The individual values obtained from these runs have already been used. In Table XXI we have recorded the observed sedimentation ratios as well as the ratios calculated from the mean values of the different types of the sedimentation constant.

TABLE XX

Mammalia: Insectivora, Rodentia, Carnivora, Ungulata, Primates

Pigment enclosed in corpuscles. Type of pigment, hemoglobin. Solvent, 1 per cent NaCl + CO. Centrifugal force 210,000–330,000 times gravity.

Origin of blood	Dilution of blood	Thickness of column of solution	$s_{20°} \times 10^{13}$
		cm.	
Erinaceidæ:			
Erinaceus europæus L.			
Upsala, Sweden	20	0.3	4.42
	20	0.3	4.57
Leporidæ:			
Oryctolagus cuniculus L.			
Upsala, Sweden	20	0.3	4.27
	20	0.3	4.44
Felidæ:			
Felis domestica Schreber			
Upsala, Sweden	30	0.3	4.26
	30	0.3	4.17
Canidæ:			
Canis familiaris L.			
Upsala, Sweden	25	0.3	4.29
	60	0.6	4.11
Equidæ:			
Equus caballus L.			
Upsala, Sweden		0.6	4.40
			4.42*
Bovidæ:			
Bos taurus L.			
Upsala, Sweden	30	0.3	4.18
	30	0.3	4.36
Cercopithecidæ:			
Cercopithecus sp.			
W. Africa	40	0.6	3.94
	20	0.3	3.86
	27	0.3	4.22
Hominidæ:			
Homo sapiens			
	—	0.6	4.54
	20	0.3	4.42
			Mean 4.29

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* Mean of four determinations by K. O. Pedersen.

The observed and calculated ratios agree within the limits of experimental error. From the observed ratios and the sum of all the absolute values of sedimentation constants a set of relative values may be calculated. In Table XXII these constants together with the absolute values found by us and those determined by other investigators are given.

Table XXI

Observed and calculated sedimentation ratios for respiratory pigments dissolved in the blood of the invertebrates, and showing two boundaries when subjected to the mixture test.

Species	Sedimentation Ratio		Deviation from calc. ratio
	Observed	Calculated	*per cent*
Helix pomatia/Nereis virens	1.72	1.75	−1.7
Helix pomatia/Sepia officinalis	1.72	1.75	−1.7
Helix pomatia/Octopus vulgaris	1.86	1.99	−7.0
Helix pomatia/Planorbis corneus	2.92	2.95	−1.0
Lumbricus terrestris/Nereis virens	1.08	1.08	±0
Lumbricus terrestris/Arenicola marina	1.07*	1.08	−0.9
Nereis virens/Planorbis corneus	1.67	1.68	−0.6
Sepia officinalis/Octopus vulgaris	1.12	1.14	−1.8
Sepia officinalis/Homarus vulgaris	2.44	2.43	+0.4
Rossia oweni/Octopus vulgaris	1.13	1.14	−0.9
Octopus vulgaris/Planorbis corneus	1.51	1.48	+2.0
Octopus vulgaris/Homarus vulgaris	2.29	2.13	+7.5
Planorbis corneus/Homarus vulgaris	1.33	1.44	−8.3
Calocaris macandreæ/Homarus vulgaris	1.50	1.44	+4.2
Homarus vulgaris/Pandalus borealis	1.41	1.39	+1.4
Homarus vulgaris/Palæmon fabrici	1.39	1.39	±0
Homarus vulgaris/Eupagurus bernhardus	1.29	1.39	−7.8
			mean dev. −0.95

* Determined by Inga-Britta Eriksson-Quensel.

Discussion of Results

One of the most striking points borne out by the above investigation is, perhaps, the fact that low sedimentation constants were always found for the pigments enclosed in red blood corpuscles. Only four different values were observed, viz., 2.1, 2.6, 3.5 and 4.4×10^{-13}, probably corresponding to molecules of mass $1/2 \times 34{,}500$, $1 \times 34{,}500$, a mixture of

Table XXII

Absolute and relative values of the seven highest sedimentation constants of blood pigments.

Standard species	$s_{20^\circ} \times 10^{13}$				
	Mean absolute value				Relative value
	Present invest.	No. determinations	Other invest.	No. determinations	
Helix pomatia	100.1	58	100.1	39*	98.3
Lumbricus terrestris	61.7	13	60.9	14†	61.8
Nereis virens	57.0	61	57.4	15†	57.1
Octopus vulgaris	50.1	13	51.7	29†	51.4
Planorbis corneus	33.9	25	33.7	11†	33.9
Homarus vulgaris	23.5	35	23.1	11†	23.6
Pandalus borealis	16.9	19	16.1	18†	17.1

* Determinations by Inga-Britta Eriksson-Quensel and K. O. Pedersen.
† Determinations by Inga-Britta Eriksson-Quensel.

both, and 2 × 34,500 containing 1, 2, and 4 atoms of iron respectively. The first three values are represented in the invertebrates and in the lowest class of the vertebrates, the Cyclostomata, while the last value is found exclusively in the higher classes of the vertebrates. Among the amphibians and reptiles a higher component of constant 7.1×10^{-13}, probably corresponding to a molecule built up of two normal hemoglobin molecules, was sometimes observed. The reddish-brown corpuscles found in the cœlomic fluid of the sipunculoid worms contain an entirely different protein, the hemerythrin, which has, however, a sedimentation constant of the same order of magnitude as the hemoglobin of the higher vertebrates. From the standpoint of blood kinship it would seem reasonable to assume that the vertebrates have developed from some invertebrate group possessing blood corpuscles and characterized by a red respiratory pigment of the same sedimentation constant and molecular weight. As a matter of fact, the lowest class of the vertebrates, the Cyclostomata, possesses a respiratory blood protein which with regard to sedimentation constant resembles closely the pigment contained in the erythrocytes of the capitellide worms. It is of interest to note that H. E. Jordan (1933) arrives at a similar conclusion from the consideration of histological data.

The blood pigments which occur dissolved in the plasma are as a rule characterized by high sedimentation constants and consequently by high molecular weights. The only exception so far observed is the red pigment of the *Chironomus* larvæ, which has a constant identical with that of the red pigment contained in the erythrocytes of the capitellide worms. It might seem tempting to put forward the hypothesis that the giant pigment molecules dissolved in the blood plasma play, to a certain extent at least, the rôle of blood corpuscles. On the other hand, it must be borne in mind that even the largest of these molecules, that of the Gastropoda hemocyanin, has a diameter of only 1/200 of that of a red blood corpuscle.

The remarkable constancy of the mass and shape properties of the molecules of the respiratory proteins within closed animal groups, as expressed by the identity of the sedimentation constants, is probably a measure of the similarity of the chemical processes which lead to the formation of these pigments. To a certain extent, therefore, identity of sedimentation constant may be taken as a criterion of biological kinship. From this point of view it is of interest to note that the hemocyanins of the very old arthropod genera *Limulus* and *Euscorpius* have the same sedimentation constant, viz., 34×10^{-13}. Only two other genera, the crustacean *Calocaris,* the blood of which contains hemocyanin, and the red-blooded snail *Planorbis* have the same constant.

One must, however, be very careful when trying to trace relationships between the various classes of animals from the properties of their blood pigments. The above investigation has shown that the number of different sedimentation constants is very restricted (so far only 14 have been observed) and that the same constant occurs in respiratory proteins containing different active groups. It is therefore not unprobable that the same constant may sometimes be represented in animal classes which could hardly be looked upon as nearly related. As an example of this kind, one might mention the erythrocruorin of *Lumbricus* and the hemocyanin of *Tonicella,* which have the same sedimentation constant, viz., 61×10^{-13}.

The observations about the behaviour of the blood pigments near the borders of the pH stability regions as well as the systematic study of the entire stability ranges and the determination of their molecular weights from sedimentation equilibrium measurements of some of these proteins, now being carried out by Mrs. I.-B. Eriksson-Quensel, seem to indicate that the blood protein molecules of high sedimentation constants break up into definite units at certain critical pH values and that these dissociation products are identical with the blood proteins of lower molecular weight as far as the mass and shape properties go. It seems, therefore, that only a few molecular masses are stable and that it would depend upon the composition of the molecule with regard to the various amino acids or other constituents and upon the actual pH of the solution whether one or the other of the different possibilities is realized.

A detailed investigation of the isoelectric points of the respiratory proteins by Dr. K. O. Pedersen (1933) of this laboratory has shown, however, that, as a rule, each species is characterized by a special value of the isoelectric point of its blood pigment. The chemical composition of the respiratory protein therefore varies from one species to the other even in cases where the sedimentation constants are identical. No relationship has so far been found between the position of the isoelectric point and the mass of the molecule. At the present time we are not able to predict what sedimentation constant a respiratory protein of known chemical composition would have, but it is believed that this will become possible with increasing knowledge of the constitution of the protein molecule.

The investigation has been made possible by financial help from the Rockefeller Foundation, the Nobel Foundation, the Foundation Therese och Johan Anderssons Minne and from the Astra Co. Most of the marine species were put at our disposal by the Zoölogical Station at Kristineberg, Sweden. We wish to express our sincere thanks to the Prefect, Professor E. Lönnberg, for his kindness in allowing these collections to be made for us. To the Director of the Station, Dr. G. Gustafson, we are greatly indebted for his constant readiness to provide us with the desired species, and for the care which he has taken in finding and collecting them for us. A number of samples of blood from marine animals were kindly sent us from the Zoölogical Station at Naples. We are most grateful to Professor R. Dohrn and Dr. L. Califano for this courtesy. We likewise wish to extend our hearty thanks to Dr. A. C. Redfield, of the Oceanographic Institution at Woods Hole, Mass., for his kindness in helping us to procure samples of blood from some especially interesting marine species not found in Europe. Professor S. Ekman, Dr. I. Arwidsson of the Department of Zoölogy, Upsala, and Professor N. Holmgren of the Department of Zoölogy, Stockholm, have given us valuable help with regard to land and freshwater animals.

SUMMARY

1. A systematic study of the sedimentation constants of the respiratory blood proteins throughout the animal kingdom has been carried out by means of the ultracentrifugal method.

2. Respiratory proteins enclosed in corpuscles have low sedimentation constants. The following values were found for erythrocruorin: capitellide worms and Cyclostomata, 2.1×10^{-13}; glyceride worms and lamellibranchians, 3.5×10^{-13}; holothurians, 2.6×10^{-13}. Hemoglobin characterized by the sedimentation constant 4.4×10^{-13} occurs only in

the five higher classes of the vertebrates, viz., Mammalia, Aves, Reptilia, Amphibia, Pisces. Among the reptiles and amphibians an association product of hemoglobin with sedimentation constant 7.1×10^{-13}, probably representing a doublet, was often found. This product was never observed in the other classes of the vertebrates.

3. Respiratory proteins dissolved in the plasma have, as a rule, high sedimentation constants. The only exception is the erythrocruorin of the *Chironomus* larvæ, which has the constant 2.0×10^{-13}. All polychæte worms and hirudineans with dissolved pigment have the constant 57.5×10^{-13} (erythrocruorin and chlorocruorin). A dissociation product of constant 11.7×10^{-13} (probably 1/16 of the normal molecule) is sometimes found. The oligochæte worms have a variety of erythrocruorin of slightly higher constant, 61.9×10^{-13}. The crustaceans show as a rule two sedimentation constants, 16.9 and 23.5×10^{-13}. The former one, which occurs both in erythrocruorins and hemocyanins, probably represents a molecular weight of one-half of the latter. Species characterized by the latter constant have hemocyanin and give a mixture of both constants in alkaline solution, thus demonstrating the dissociation into half molecules. One crustacean species (*Calocaris macandreae*) has a hemocyanin constant of 34.0×10^{-13}. This sedimentation constant is also characteristic of the hemocyanin of the scorpion (*Euscorpius carpaticus*). The xiphosuran (*Limulus polyphemus*) has a hemocyanin of the same constant, but its blood contains two more hemocyanin varieties of constants 16.5 and 59.1×10^{-13} and a dissociation product of constant 6×10^{-13}. The erythrocruorin of the gastropods has the same constant, 33.8×10^{-13}. The normal hemocyanin of the gastropods, on the other hand, is characterized by the constant 100.1×10^{-13}. It forms an association product of constant 133.6×10^{-13} and two dissociation products of constants 16.2 and 61.9×10^{-13} (probably 1/2 and 1/8 of the normal molecule respectively). The constant 60.9×10^{-13} is also found in the amphineuran hemocyanin (*Tonicella marmorea*). The cephalopods show two constants, 56.2×10^{-13} for the decapods and 50.1×10^{-13} for the octopods. The latter constant has not been observed in any other place and is the only sedimentation exclusively characteristic of an animal group.

4. The significance of the data collected in the present investigation cannot be fully understood until a sufficient number of pH-stability curves and sedimentation equilibrium measurements have been made on respiratory proteins. The few determinations of this kind so far available, however, seem to indicate that all native proteins form a closed system in which only a very limited number of mass and shape types

are stable. Reversible association and dissociation reactions take place easily when the pH of the solution is slightly changed. Within a well-defined animal group all species have as a rule respiratory pigments of the same sedimentation constant (or constants) and dissociate in a similar way. Biological kinship, therefore, is usually accompanied by identity in the sedimentation constants. On the other hand, owing to the small number of different constants possible, the same constant must of necessity occur in different animal groups.

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THE MALE SOMATIC CHROMOSOME GROUP IN SCIARA PAUCISETA[1]

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Introduction

The present study is designed to furnish additional evidence bearing on the general problem of sex determination in *Sciara*. It is concerned particularly with two features. In the first place, evidence is given showing that in *Sciara pauciseta* Felt there are seven chromosomes in the male soma instead of eight as in the female soma, although the germ-line chromosome groups are alike in the two sexes. In the second place, it is shown that this evidence adds support to the hypothesis that the "precocious"[2] chromosome in *Sciara* is a sex chromosome (Metz, 1929, 1930).

Earlier evidence bearing on the immediate problem may be summarized briefly as follows:

1. In all species of *Sciara* thus far adequately studied, the chromosome group of the germ-line in both sexes (*i.e.*, spermatogonia and oögonia) consists of symmetrical pairs of chromosomes—except for variations in the number of "limited" chromosomes which are found only in the germ-line and hence do not concern us at present.[3]

2. The chromosome group of the female soma in these species is regularly composed of four symmetrical pairs.

3. The chromosome group of the male soma in *Sciara coprophila* is composed of three symmetrical pairs of chromosomes and an unpaired chromosome. The eighth chromosome found in the female soma is eliminated during cleavage in the somatic line of the male. (Metz, 1931*a*; Du Bois, 1932.)

4. Genetic evidence indicates that this unpaired chromosome is regularly the maternal X-chromosome, and that, therefore, it is the paternal

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[1] This investigation has been aided by a grant from the National Research Council, Committee for Research in Problems of Sex; grant administered by C. W. Metz.

[2] For explanation of "precocious" chromosome, see No. 6 under the "earlier evidence" in the Introduction.

[3] See Metz, Moses, and Hoppe, 1926; Metz, 1926, 1931*a* and *b*; Metz and Schmuck, 1931*b*.

sex chromosome which is regularly eliminated from the male soma. (Metz, *loc. cit.*; Metz and Schmuck, 1931*a*.)

5. Available evidence makes it seem probable that this paternal sex chromosome is an X, not a Y, and that sex in the male is determined by the constitution of the soma. It has not been possible, however, to demonstrate this.

6. In all species of *Sciara* in which spermatogenesis has been studied, the second spermatocyte division exhibits peculiarities resulting in the transmission to all sperms of both halves of one chromosome called the "precocious" chromosome. It has been suggested that this chromosome is a sex chromosome and that its behavior may have a bearing on sex determination (Metz, 1930, 1931*b*).

7. So far as known, all species of *Sciara* possess four pairs of chromosomes in the female somatic cells. The groups are not all alike, however, in respect to shape and size of the chromosomes. Some species possess three pairs of rod-like and one pair of V-shaped chromosomes, while in others there are two pairs of rods and two pairs of V's. In species of the first group the "precocious" chromosome is regularly one of the rod-like members, and in those of the second it is regularly one of the V-shaped members (Metz, Moses, and Hoppe, *loc cit.*; and unpublished evidence of C. W. Metz).

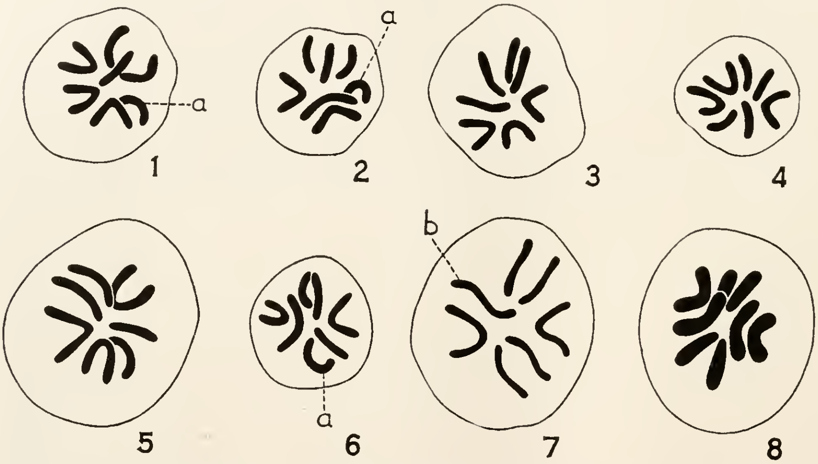
It is evident from the above résumé that if the "precocious" chromosome is a sex chromosome as postulated, it should regularly agree in size and form with the one eliminated from the male somatic nuclei, and hence the examination of species having different types of "precocious" chromosomes should furnish evidence for or against the hypothesis under consideration. Of course, the method can give support to the hypothesis only in an indirect manner, but it might provide conclusive contrary evidence if the hypothesis is incorrect. In the case of *Sciara coprophila,* already noted, there is agreement between the "precocious" chromosome and the sex chromosome, both of which are rod-like. The present study deals with a representative of the other type and shows that there is also an agreement where the "precocious" chromosome is V-shaped.

OBSERVATIONS

The present observations were made on young larvæ. The specimens were dissected, fixed in Flemmings' fluid (strong), and stained by the Feulgen method. This stain gives excellent results, since only the chromatin becomes colored. The migratory cells of the nervous system, as well as other somatic cells, were used. The former are particularly good because, although few in number, they are large and the mitotic figures are clear, with the chromosomes sharply defined.

The female somatic group in this species consists of two pairs of rod-shaped and two pairs of V-shaped chromosomes. An example is shown in Fig. 8. (See also Metz, 1926, Fig. 34.) The shapes are, of course, determined by the point of spindle fiber attachment, which is terminal in the rod-like and median in the V-shaped members. A further distinction is seen in the size of the two types—the V-shaped chromosomes being considerably longer than the rod-like ones in most cases.

The male somatic groups, obtained from a large number of individuals, all show seven chromosomes—two pairs of rods, one pair of V's and an unpaired V. Seven such groups are shown in Figs. 1 to 7, from camera lucida drawings made from sectioned material. Frequently a drawing does not show accurately the shape of the chromosomes, since they may not be flat on the metaphase plate. Figures 3, 4, and 5 show most clearly the three V's and four rods. Careful detailed studies of the chromosome marked "*a*" in Figs. 1, 2 and 6 show that it is a rod and not a V as might be thought at first glance. In Fig. 7 the chromosome marked "*b*" is a V, although in the drawing it appears nearly straight.



Figs. 1–8. Somatic chromosome groups of *Sciara pauciseta* Felt. Figures 1–7 from male somatic cells. Figure 8 from somatic cell of female (ovarian follicle cell). Drawings for Figs. 1–7 made by Miss Louise H. Buck.

Although the actual process of chromosome elimination during cleavage has not been observed in the present species, it seems clear from the evidence presented above, together with that cited from earlier studies on spermatogonia and female somatic chromosome groups, that a V-shaped sex chromosome is eliminated from male somatic cells here just as a rod-like one is eliminated in *Sciara coprophila*. Since in the present species the " precocious " chromosome is V-shaped, it is clear that the evidence, so far as it goes, supports the hypothesis that the " precocious " chromosome is a sex chromosome.

The author is deeply indebted to Dr. C. W. Metz for his valuable help in working out this problem.

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EFFECTS OF CENTRIFUGAL FORCE ON THE ECTOPLASMIC LAYER AND NUCLEI OF FERTILIZED SEA URCHIN EGGS

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In previous studies (E. B. Harvey, 1932, 1933*a*, 1933*b*) of sea urchin eggs stretched and broken apart by centrifugal force, it was found that unfertilized and fertilized eggs behave quite differently. The former form dumb-bells and pull apart as spheres of very definite size, while the latter during a certain period (monaster stage) form long streamers which break irregularly. A more extensive study has now been made of the fertilized eggs of some of the European sea urchins in which, owing to the clear, unpigmented protoplasm, the nuclear phenomena can be seen with great clearness in the living egg. It is possible by centrifugal force to separate the male and female pronuclei so that they come to lie either in different regions of an elongated egg, or in completely separated fragments. A study of the development of these eggs and fragments is presented in this paper, together with observations on the outermost layer of the fertilized egg (the ectoplasmic layer), which is actually peeled off the egg by centrifugal force. It has also been found that if eggs are centrifuged just before cleavage, the cleavage plane may come in without any relation to the new position of the mitotic figure.

ECTOPLASMIC LAYER

The ectoplasmic or hyaline plasma layer of the sea urchin egg is a thin colorless layer appearing on the surface of the egg following fertilization, and is quite distinct from the fertilization membrane. It has an important function in holding the blastomeres together; the layer is disorganized and the blastomeres fall apart in the absence of calcium (Herbst, 1900; Gray, 1924, 1931; Moore, 1930). Some investigators (Ziegler, 1903; Gray, 1924, 1931; and Just, 1928) have considered it of importance in the mechanics of cell division, albeit for different rea-

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[1] I wish to express my appreciation of the interest and courtesy of Dr. Reinhard Dohrn, Director of the Stazione Zoologica, during my stay in Naples, and of the facilities given me by the Jacques Loeb Memorial Table of the Rockefeller Institute, New York.

sons. The characteristic heaping of the layer at the equator at the beginning of cleavage would certainly lead one to believe that it is in some way concerned with the cleavage process.

In most sea urchins, the Naples species, *Paracentrotus lividus, Parechinus microtuberculatus, Sphærechinus granularis*, and the Woods Hole species, *Arbacia punctulata*, the layer, though detectable, is extremely thin (less than 1 μ) until ten or fifteen minutes after fertilization and it gradually becomes thicker until it measures 2 or 3 μ before cleavage (Fig. 1). In the Naples species of *Arbacia, A. pustulosa*, the ectoplasmic layer is well formed at the time that the fertilization membrane lifts off and even then measures 2–3 μ. It is particularly striking in this species as it makes a sharp contrast with the deeply colored egg. It is perfectly apparent here, by measuring the same egg (with a water immersion lens) before and after insemination, that the ectoplasmic layer is *added on* to the surface of the unfertilized egg. The diameter of the total egg increases 4–5 μ; this increase cannot be caused by any flattening of the egg due to gravity since the diameter of the colored portion remains the same. (See E. N. Harvey, 1933.) Glaser (1914, 1924) found (also by direct measurement) a *decrease* in diameter of the *Arbacia punctulata* egg on fertilization. In this species, however, it is to be noted that the ectoplasmic layer is extremely thin at this time so that any change in diameter due to it could scarcely be measured. His results have been questioned by Chambers (1921) and others. The layer seems to be, morphologically, in the nature of an extracellular membrane, rather than an integral part of the egg protoplasm, though this in no way detracts from its physiological importance. The behavior of the layer when subjected to centrifugal force leads to the same conclusion.

When the eggs of any of these sea urchins are centrifuged at any time after ten minutes following insemination, in a sucrose solution of the same density and tonicity as the eggs (in order to keep them suspended), at a centrifugal force of about 5000 × g, the ectoplasmic layer is centrifuged off the egg, usually in the form of a ring. It is best observed in eggs which have a large perivitelline space such as *Parechinus* (20 μ) and *Paracentrotus* (12 μ). When the eggs are first removed from the centrifuge, the ring is seen encircling the slightly tapering heavy pole of the egg, and the characteristic ectoplasmic layer investing the surface of the normal fertilized egg is lacking (Fig. 2, Photograph 1). As the egg becomes spherical, the ring slips off and lies free in the perivitelline space (Fig. 3). It remains here through cleavage and can be seen attached to the blastula (Fig. 4) even after it has become free-swimming, but eventually drops off. The ring is not always perfect, but is often incomplete or broken or has vacuolated or thinner areas (Fig. 5). If eggs are centrifuged 7–10 minutes after fertilization, the ring is very narrow; if centrifuged before that, the ring does not form since there is an insufficient amount of ectoplasmic material. It may be centrifuged off also in the 2- and 4-cell stages, but is then of smaller size and more irregular shape (Fig. 6). The ring (and also the intact ectoplasmic layer) does not stain with any intravitam dyes; in such dyes as methylene blue, brilliant cresyl blue, and neutral red, it remains quite colorless, forming a sharp contrast to the heavily staining granular protoplasm. The ectoplasmic layer of the *Arbacia* egg (both *A. punctulata* and *A. pustulosa*) can also be thrown off, but owing to the small perivitelline space (1–5 μ), it is usually difficult to observe unless the fertilization membrane is broken at the centripetal pole and has slipped partly off, leaving a large space between it and the surface of the egg at the centrifugal pole. (Photograph 2.) In *Arbacia punctulata* it usually appears as a crescent rather than a ring. After the ectoplasmic layer from any of the eggs with intact fertilization membranes has been centrifuged off, it is gradually reformed on the surface of the egg, so that in about thirty minutes it looks almost the same as in uncentrifuged eggs, and after cleavage the blastomeres cohere. Perfectly normal plutei develop from these eggs in as large a percentage as from the controls. The ectoplasmic layer must also be replaced during the development of isolated blastomeres separated in calcium-free sea water, since, when returned to sea water, they may form perfect dwarf plutei whose cells cohere. It will be noted that the ectoplasmic layer differs markedly in this respect from the fertilization membrane, which is formed only once and is never replaced when once removed.

The ring-formation from the ectoplasmic layer, as described above, takes place in eggs centrifuged in an isotonic sucrose solution brought to the same density as the eggs by the addition of sea water; after centrifuging, the eggs were immediately returned to sea water. If the eggs

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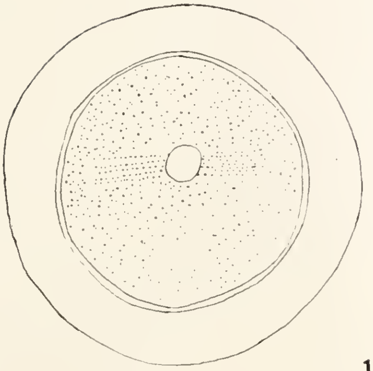
PLATE I

Parechinus microtuberculatus

1. Normal egg 30 minutes after insemination, showing ectoplasmic layer.
2. Egg centrifuged 30 minutes after insemination (for 3 minutes at 5,000 × g), showing ectoplasmic ring encircling heavy pole of elongate egg.
3. Same egg 10 minutes later; ectoplasmic ring lying in perivitelline space.
4. Same egg, early blastula; ectoplasmic ring still present.
5. Various forms of ectoplasmic ring when centrifuged off before first cleavage.
6. Various forms of ectoplasmic ring centrifuged off during 2-cell stage.
7. Egg kept and centrifuged in calcium-free medium, then put into sea water. Ectoplasmic material has precipitated in perivitelline space as small refringent bodies. Fertilized at 10:37; placed in calcium-free medium at 10:42; centrifuged from 10:47–10:51; then put in sea water; drawn at 10:55.

PLATE I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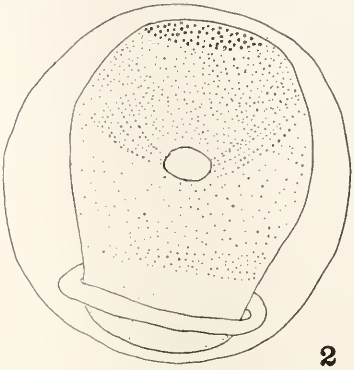
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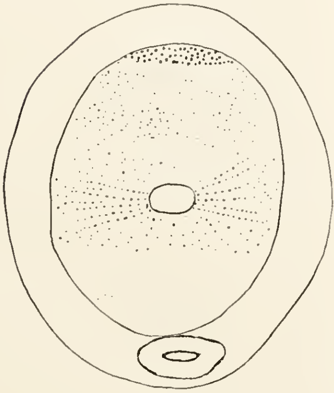
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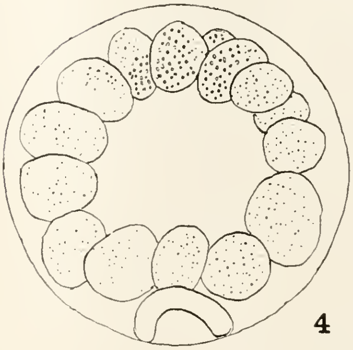
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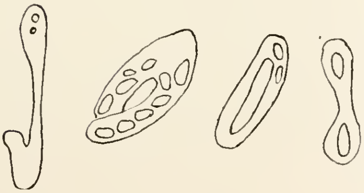
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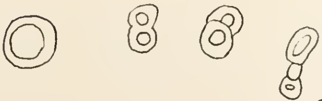
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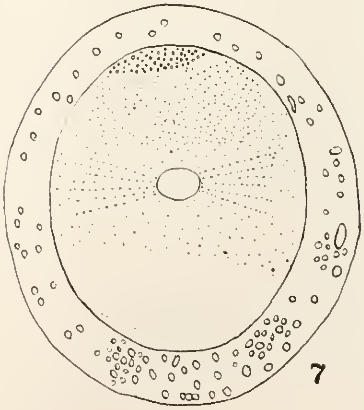
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are put in *calcium-free* sea water five minutes after fertilization (*i.e.*, time to allow the fertilization membrane to harden), in order to disintegrate the ectoplasmic layer, and are then centrifuged in a mixture of this solution and isotonic sugar (same density as the eggs), the ring does not form. If the eggs after removal from the centrifuge are left in the same solution (*i.e.*, minus Ca) or transferred to calcium-free sea water, the perivitelline space remains perfectly clear and there is no ectoplasmic layer on the surface of the eggs. If the eggs, on removal from the centrifuge, are put in normal sea water, the perivitelline space becomes filled with many small refringent spherical or oval bodies (Fig. 7), the precipitation product of the ectoplasmic material in the presence of calcium. On return to calcium-free sea water, these are again dissolved and can be precipitated again in the presence of calcium. The ectoplasmic material, after being centrifuged off as a ring, may also be dissolved in calcium-free sea water and be precipitated again as scattered spherules when returned to sea water. Similar refringent spheres arising from the ectoplasmic layer have been described in eggs treated with acid sea water by Gray (1924, 1931) and by Moore (1928, 1932). The ectoplasmic layer, according to these two authors, reacts chemically like a calcium proteinate. Whatever its exact chemical nature, my experiments show that the ectoplasmic layer is a very definite external layer or membrane, highly unstable in the absence of calcium, easily peeled off by centrifugal force, and readily reformed under certain conditions after removal.

PROTOPLASM

The effects of centrifugal force on the protoplasm of the fertilized eggs of *Parechinus*, *Paracentrotus*, *Sphærechinus*, and *Arbacia pustu-*

Photographs 1–8

1. *Parechinus microtuberculatus.* Fertilized at 11:42; centrifuged from 11:52–11:58; taken at 11:59. Note ectoplasmic ring encircling heavy (small) pole of egg.

2. *Arbacia punctulata.* Centrifuged 8 minutes after fertilization. Ectoplasmic crescent within broken fertilization membrane.

3. *Parechinus microtuberculatus.* Fertilized at 10:15; centrifuged from 10:18–10:22 (5,000 × g). Eggs fragmented in many small pieces.

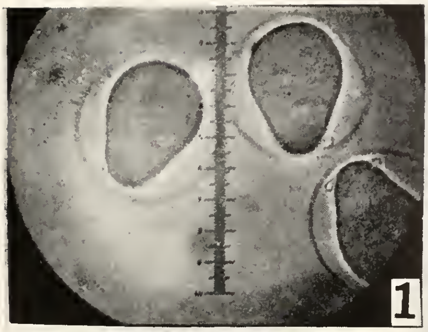
4. *Sphærechinus granularis.* Centrifuged 21 minutes after fertilization for 3 minutes (5,000 × g). Streamer stage, great elongation.

5. *Sphærechinus granularis.* Much elongated egg breaking up into small fragments.

6. *Parechinus microtuberculatus.* Fertilized at 8:50; centrifuged from 9:01–9:05. Taken at 9:15. Note bulge in egg where male aster is.

7. *Parechinus microtuberculatus.* Centrifuged 9 minutes after fertilization. Female nucleus below oil, ♂ nucleus in center; note bulge here.

8. *Parechinus microtuberculatus.* Fertilized at 10:15; centrifuged from 10:23–10:27. Egg more contracted; ♂ and ♀ nuclei present.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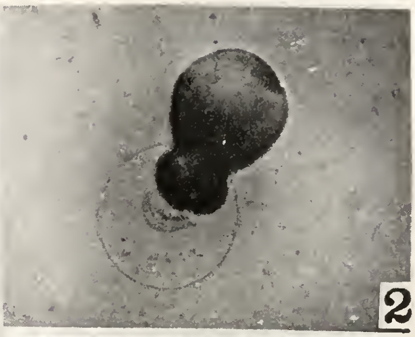
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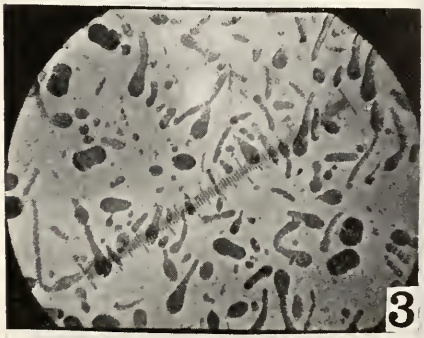
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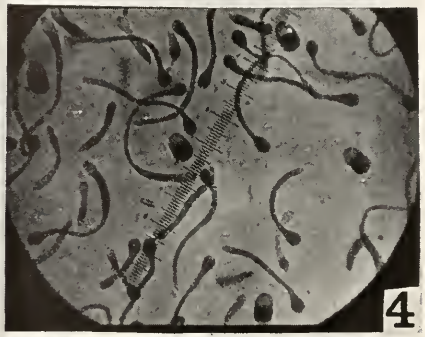
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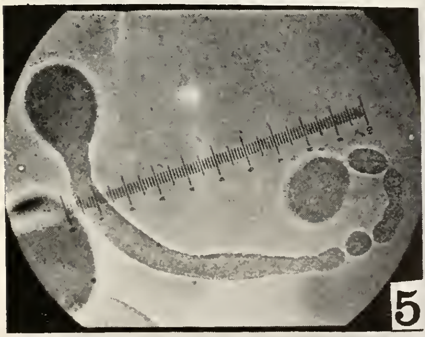
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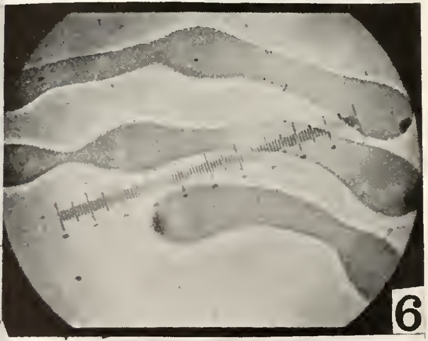
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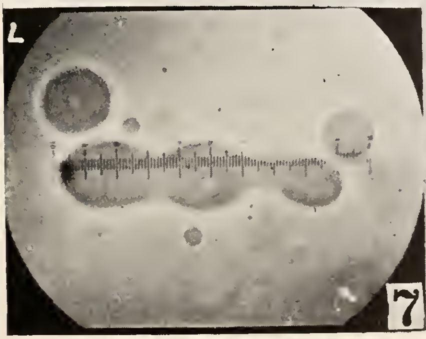

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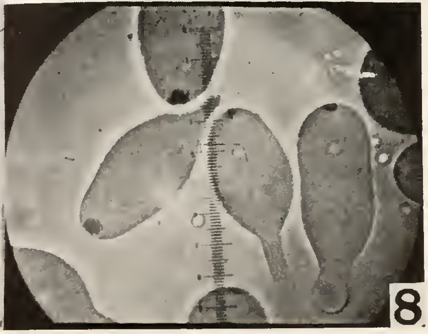
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losa have been found to be practically the same as described for *Arbacia punctulata* (E. B. Harvey, 1933*b*). There is a period soon after fertilization (about 1½ to 6 minutes at 18° C.) when the eggs break very readily into many small pieces (Photograph 3); even slight shaking in the case of *Parechinus* and *Paracentrotus* at this period is sufficient to thoroughly fragment the eggs. The fertilization membrane at this time is elastic and easily broken; it sometimes stretches to twice its original diameter before breaking. After about six minutes it becomes quite tough and inelastic, and must be removed from the eggs (at 2 minutes after insemination by shaking) for experiments with the later stages. During or before the monaster stage (6–30 minutes after insemination, the time varying with the different species), the eggs elongate greatly, forming long streamers sometimes fourteen times the original diameter of the egg (Photographs 4–6); the streamers of the *Parechinus* and *Paracentrotus* egg are longer than those of the *Sphærechinus* egg. The streamers retract very quickly immediately upon removal from the centrifuge; a *Parechinus* egg which was stretched to 1300 μ retracted to 455 μ in five minutes. After the first few minutes they retract more gradually, often becoming spherical or nearly so, within an hour; they frequently break into small fragments, especially in the intermediate zone between the light and heavy poles which tend to remain somewhat intact (Photographs 4, 5). During the streak stage, the eggs elongate less and just before cleavage very little. The fertilized eggs of *Parechinus* and *Paracentrotus* break at all stages *more* readily than unfertilized eggs of the same batch centrifuged at the same time; the fertilized eggs of *Sphærechinus* break at all stages *less* readily than control unfertilized eggs. *Arbacia punctulata* is intermediate in this respect (E. B. Harvey, 1933*b*). In all the forms, the fertilized

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PLATE II

Parechinus microtuberculatus

8. Normal egg showing size of pronuclei at time of union; 14 minutes after insemination.

9. Egg fertilized at 9:45; centrifuged (5,000 × g) from 9:55–10:00; drawn at 10:10. Male nucleus measures 8 μ; ♀ nucleus 13 μ.

10. Same egg drawn at 10:25. Male and ♀ nuclei measure 13 μ.

11. Same egg drawn at 10:35. Male nucleus measures 19 μ; ♀ nucleus 8 μ. Note nucleolus in ♂ nucleus.

12. Same egg drawn at 10:40. Nuclei have fused and measure 20 μ.

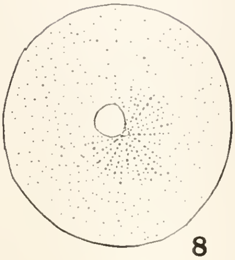
13. Same egg drawn at 11:45. Cleavage amphiaster.

14. Another egg. Fertilized at 11:28; centrifuged from 11:37–11:41; drawn at 12:07. Male nucleus forms amphiaster before union with ♀.

15. Another egg. Fertilized at 10:05; centrifuged from 10:12–10:16; drawn at 11:15. Male nucleus forms amphiaster, ♀ monaster.

16. Same egg at 11:22; tripolar cleavage.

PLATE II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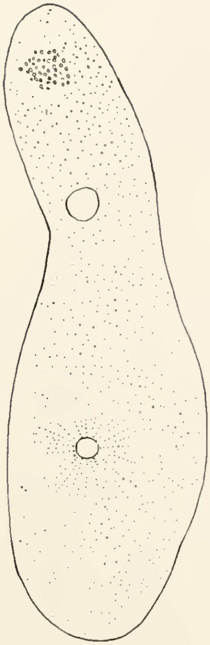
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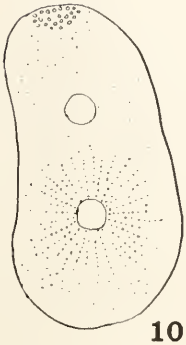
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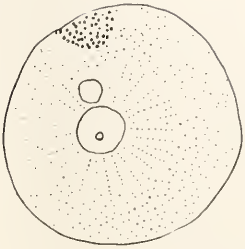
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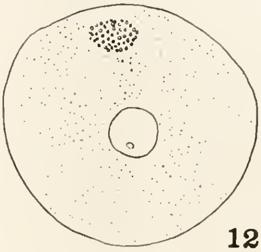
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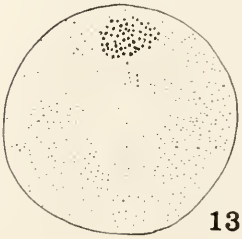
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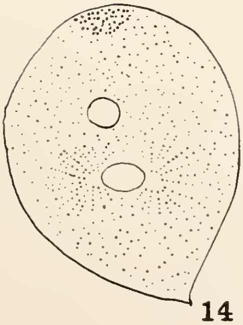
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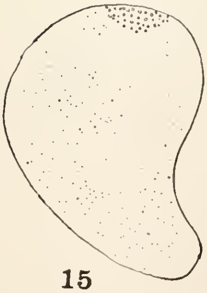
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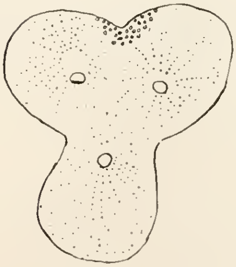
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eggs stratify less readily than the control unfertilized eggs, showing that they are more viscous; this difference is slight soon after fertilization but becomes more marked in later stages. As has been pointed out in the case of *Arbacia punctulata,* the greater ease of breaking must be due to surface differences between the fertilized and unfertilized eggs. The differences cannot, however, be due to the ectoplasmic layer, since this is scarcely formed at the time of maximal fragmentation and in later stages is thrown off by the centrifugal force as previously described.

NUCLEI

In the eggs greatly elongated by centrifugal force (during or just before the monaster stage), the two pronuclei are thrown apart, the female pronucleus going to the light pole and the male to the heavy pole. The presence of the male nucleus in the heavy end of the eggs (of *Parechinus, Sphærechinus,* and *Paracentrotus*) is often made apparent first by the sperm aster. A pronounced bulging of the egg in this region (Photographs 6, 7) lends support to the generally accepted view that asters are of considerable rigidity and form regions of greater gelation. That the two nuclei should go to different poles is not surprising in view of the fact that at this time the male nucleus consists practically entirely of chromatin material in a very condensed form, whereas the female nucleus consists of the same material together with a large amount of other more fluid material. The spermatozoa are themselves heavier than the entire egg since they are thrown by centrifugal force to the bottom of a tube in the same medium in which the eggs remain suspended.

In normal uncentrifuged sea urchin eggs, the two pronuclei unite soon (6–15 minutes) after insemination while still very unequal in size (Fig. 8). By centrifuging the eggs, the two nuclei become so widely separated that it takes considerable time (an hour or more) for them to come together, which they do both because of the contraction of the egg protoplasm and by movement on their part through the protoplasm. During this time, the male nucleus increases enormously in size, so that it becomes not only of equal size with the female but often much larger; it frequently acquires a nucleolus while enlarging (Figs. 9–12, Photographs 7, 8). There is considerable variation in the actual size attained by the two nuclei; the female nucleus of *Parechinus* is about 13 μ in diameter, increasing to 16 μ before dissolving; the male nucleus has been observed to increase to 19 μ. The discrepancy in size is often much greater owing to the fact that the female nucleus may get smaller (before disappearing) at the same time that the nearby male nucleus is getting larger (Fig. 11). The fusion nucleus of the *Parechinus* egg usually measures 16 μ at its maximum, but has been observed to increase to 22 μ.

The male nucleus does not increase greatly in size (usually under 10 μ) when it is alone in a fragment obtained by centrifuging either before or after fertilization. This is probably due to the greater packing of granules in the surrounding protoplasm in these fragments as compared with the protoplasm surrounding the male nucleus when present (together with the female pronucleus) near the centripetal pole. The large size (22 μ) sometimes attained by the fusion nucleus in the clear quarter eggs of *Arbacia punctulata* where the surrounding medium is granule-free (E. B. Harvey, 1932) lends support to this explanation.

The importance of the time factor in controlling the size relations of the two nuclei is shown in the case of the eggs of many animals, annelids, mollusks, and even the closely related *Asterias* (Wilson and Mathews, 1895). The sperm here enters or may enter the egg before the polar bodies are given off and during the delay thus caused, the sperm nucleus increases in size until it is equal to the female. The two nuclei also become of equal size in the sea urchin *Toxopneustes,* if their union is delayed by treatment with ether (Wilson, 1902).

After the female and large male pronuclei unite as above described (Fig. 12), an amphiaster is formed and the egg cleaves normally (Fig. 13). Frequently the male centrosome divides before union of the two pronuclei, so that the advancing male nucleus is accompanied by an amphiaster instead of a monaster (Fig. 14). This is the case normally in *Asterias* and many annelids and mollusks and often occurs in etherized *Toxopneustes* eggs (Wilson, 1902). This amphiaster becomes or gives rise to the cleavage amphiaster after union of the two pronuclei, and normal cleavage follows. In some cases, the female nucleus decreases in size and disappears before union with the male, and the ensuing mitotic figure is then a triaster, the female nucleus being replaced by a monaster (Figs. 15, 16). The changes taking place in the male and female pronuclei and in the accompanying asters seem to be quite independent of each other and the variations occurring normally in different species can be duplicated in one species by varying the time relations of the events; this is accomplished in these elongate eggs produced by centrifugal force.

With a greater centrifugal force, the elongate egg can be broken into two parts, the lighter fragment containing the female nucleus and the denser fragment the male nucleus (Fig. 17). In the majority of experiments with both *Parechinus* and *Paracentrotus,* continuous observation of many of these half-eggs gave the following results. The fragment containing the female nucleus does not divide (Figs. 18–21). The nucleus enlarges (to about 16 μ), disappears, a large monaster

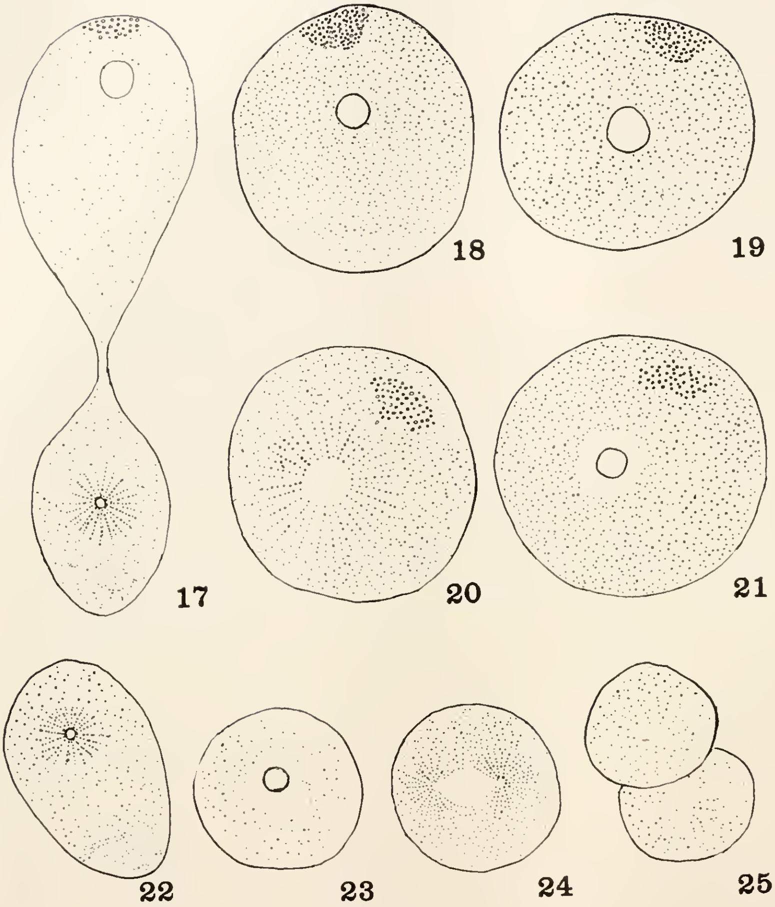

Plate III

Parechinus microtuberculatus

17. Segregation of ♂ and ♀ nuclei into separate fragments; division center associated with ♂ nucleus. Fertilized at 10:05; centrifuged from 10:12–10:16; drawn at 10:26.

18–21. Development of fragment with ♀ nucleus.

18. Egg fertilized at 9:12; centrifuged from 9:15–9:19; fragment drawn at 9:35. Nucleus measures 13 μ.

19. Same fragment at 10:10. Nucleus measures 16 μ.

20. Same fragment at 10:45. Monaster.

21. Same fragment at 10:50. Nucleus back. Monaster again like Fig. 20 at 11:10; amœboid at 12:00.

22–25. Development of fragment with ♂ nucleus.

22. Fertilized at 10:05; centrifuged from 10:12–10:16; fragment drawn at 10:30. Sperm aster.

23. Same fragment at 10:40. Larger ♂ nucleus.

24. Same fragment at 11:15. Sperm amphiaster.

25. Same fragment at 11:30. Cleavage.

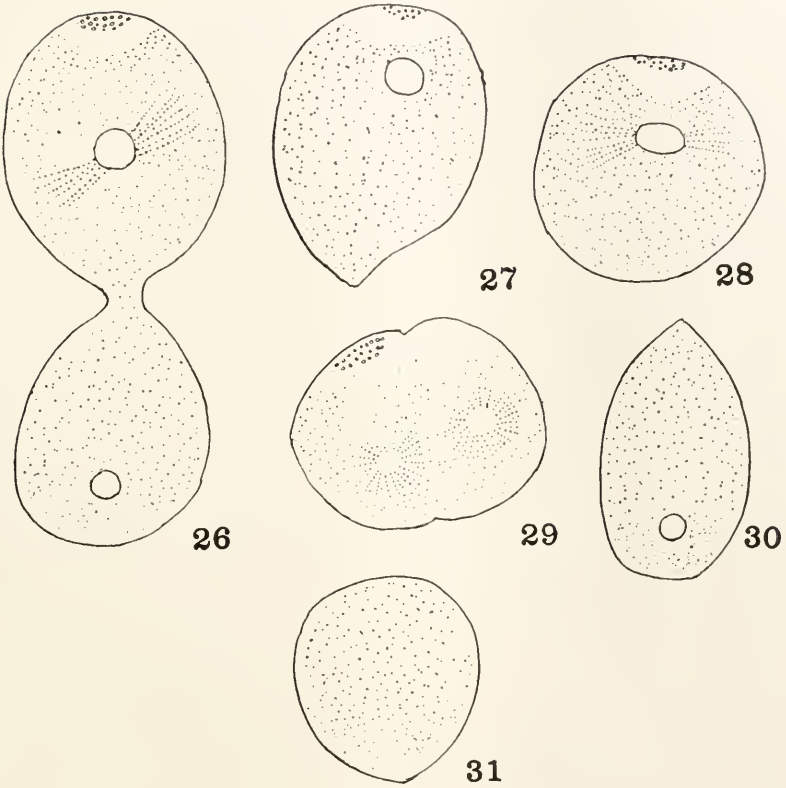

Plate IV

Paracentrotus lividus

26. Segregation of ♂ and ♀ nuclei into separate fragments; division center associated with ♀ nucleus. Fertilized at 10:20; centrifuged from 10:37–10:41; drawn at 11:00.

27–29. Development of fragment with ♀ nucleus.

27. Egg fertilized at 12:08; centrifuged from 12:25–12:30; fragment drawn at 12:40. Nucleus enlarges to $16\ \mu$.

28. Same fragment at 12:55. Rays from ♀ nucleus.

29. Same fragment at 1:20. Division.

30–31. History of corresponding fragment from same egg with ♂ nucleus.

30. Drawn at 12:40. No rays from ♂ nucleus.

31. Drawn at 12:55. Nucleus gone.

appears, the nucleus may later be reformed and again break down to form a monaster, often with a granular interior. This process may be repeated many times and finally the cell often becomes amœboid, but does not cleave. The fragment containing the male nucleus, on the other hand, develops quite normally; the nucleus enlarges (to about 10 μ), then fades out and an amphiaster appears and the cell cleaves normally (Figs. 22–25). The active division center appears to be associated with the male nucleus; and the female nucleus, lacking it, is unable to form an amphiaster. This seems curious in view of the fact that the female nucleus can function alone and form an amphiaster in parthenogenesis. It was also found that in *Arbacia punctulata,* where the egg was separated inside the fertilization membrane, the white half containing the female nucleus alone frequently developed together with the red half containing the male nucleus alone, though this was not always the case (E. B. Harvey, 1933*b*). It is interesting to find that the eggs separated by hand with a cotton thread, so that one part contained the female nucleus and the other the male (in Ziegler's (1898) experiments with the same egg, *Parechinus*), behaved in exactly the same way as these separated by centrifugal force.

In two lots of *Paracentrotus* eggs, both from the same batch, centrifuged 17 minutes after insemination, the reverse of the usual occurrence took place in many eggs, though not in all. In the whole egg partially separated, the astral rays were associated with the female nucleus and none with the male (Fig. 26). When these eggs were completely separated, the amphiaster arose in connection with the female nucleus and this fragment divided (Figs. 27–29); no rays formed around the male nucleus, but it grew smaller and disappeared and the cell never divided (Figs. 30, 31). This same lot of eggs centrifuged earlier (15 minutes after insemination) gave the usual result, the amphiaster associated with the male nucleus; when centrifuged later (19 minutes) the two nuclei were not separated. It may be that there is a critical period of short duration when the active division center, arising,

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Photographs 9–15

9. *Sphærechinus granularis.* Centrifuged in spindle stage one hour after fertilization. Note oil drops in amphiaster in clear layer.

10. *Sphærechinus granularis.* Shows division plane through oil perpendicular to stratification.

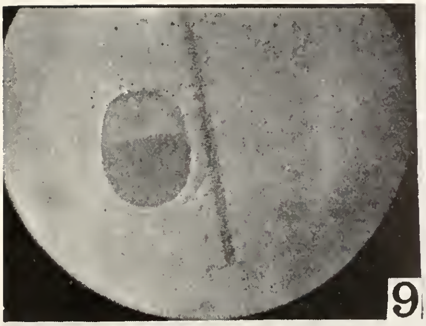
11. *Parechinus microtuberculatus.* Centrifuged just before cleavage. Note oil in astral rays.

12. Similar egg, showing division plane starting parallel with stratification and in no relation to mitotic figure, which is outlined by oil drops.

13. Similar eggs a little later. Note also disorganized ectoplasmic material in cleavage furrow.

14. *Paracentrotus lividus.* Centrifuged just before cleavage one hour after fertilization. Cleavage plane (in upper cell) has separated off clear cell which contains mitotic figure.

15. *Arbacia punctulata.* Centrifuged just before cleavage on centrifuge-microscope. Note central cell cleaved into one clear and one pigmented blastomere; the spindle is in the clear area.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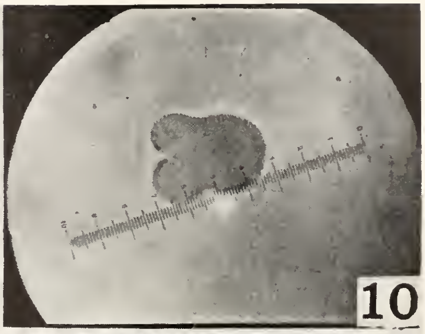
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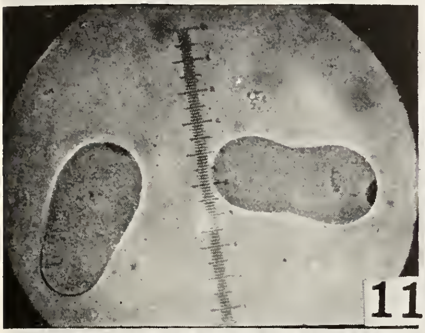
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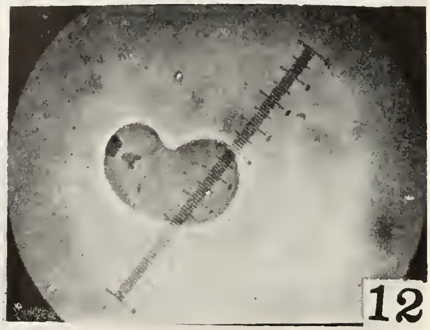
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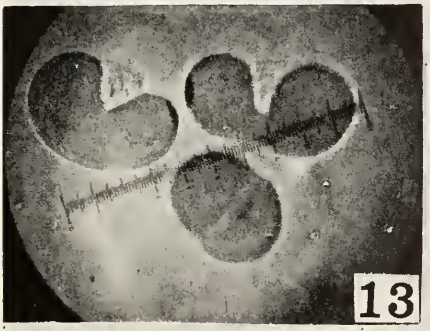
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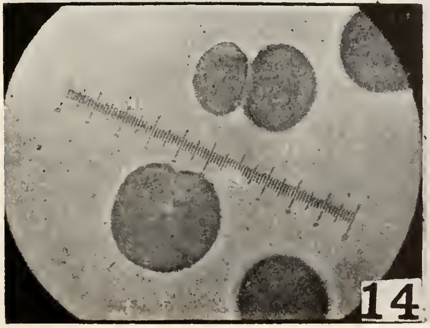
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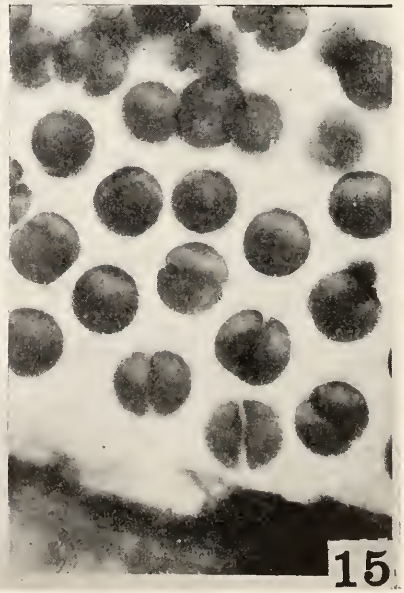
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as it usually does, in connection with the male nucleus, becomes independent of it or more closely associated with the female nucleus.

Amphiaster and Cleavage

When the eggs of any of the sea urchins are centrifuged after the amphiaster has formed, many of the oil drops are stopped in their passage to the light pole by the rays of the asters. In many cases the asters are completely outlined by the oil drops. This is particularly striking in eggs like *Sphaerechinus* and *Arbacia* where the mitotic figure lies in a perfectly clear area; this clear band is optically empty except for two small areas where the oil drops are enmeshed in the asters (Photograph 9). It is also quite striking even when the mitotic figure lies among granules as in the *Parechinus* egg (Photographs 11, 12). This demonstrates very clearly that the mitotic figure is a very definite structure consisting of more rigid or gelated material than the surrounding medium. The presence of the spindle and asters in the clear zone in fixed and stained material has been demonstrated by Spooner (1911) in the *Arbacia* egg.

If centrifuged for a sufficiently long time at a sufficiently high speed, the mitotic figure takes up its position under the oil cap with its long axis parallel with the stratification and the cleavage plane is perpendicular to the stratification (Photograph 10). This may occur even while eggs are rotating at fairly high speeds, as can be observed with the centrifuge-microscope (E. B. Harvey, 1933*b*). If, however, the eggs are centrifuged rapidly after the elongation preliminary to cleavage, the eggs orient with the long axis along the axis of centrifugal force (unless held in another position by the surfaces of the centrifuge-microscope slide). The spindle is still thrown into the light zone under the oil cap and oriented as before, but the cleavage plane may come in *parallel with* the stratification in the short axis and often in the exact position that it would have come in had the mitotic figure not been moved (Photographs 11–15). The cleavage plane thus bears no relation to the new position of the spindle, but to its former position. The change in the surface correlated with the initial location of the spindle is apparently sufficient for the formation of the cleavage plane in its original position. Whether chromosomes have been left behind and function in the formation of the cleavage plane has not been determined, as they cannot be seen in the living egg. The cleavage furrow comes in in this way, that is, without relation to the new position of the spindle, both in eggs while rotating, as can be seen with the centrifuge-microscope, and after the centrifugal force has been removed. The breaking strain due to centrifugal force also comes in this region, for at this time the eggs tend to break at or near the equator into two fairly equal parts, whereas in earlier stages they break into unequal parts. A further investigation of the relation of the mitotic figure to the cleavage plane in the transparent eggs of *Parechinus* and *Paracentrotus* will be undertaken shortly, using the centrifuge-microscope.

Later Development of Eggs and Fragments

It has been noted above that when eggs are centrifuged with the fertilization membranes intact, they develop normally although the ectoplasmic layer is thrown off; this is apparently reformed if the eggs are protected by the fertilization membrane. They do not develop in any large percentage, however, if centrifuged during cleavage; this is no doubt owing to the disturbances of the mitotic figure (and chromosomes) mentioned above. It has also been noted previously that fragments without nuclei do not develop, and fragments containing only the female nucleus do not usually develop. Whole eggs and fragments other than those mentioned may develop and form swimming blastulae and plutei. In many cases, however, loose clusters of cells are formed owing to the lack of an ectoplasmic layer. This is true for eggs centrifuged soon after fertilization when the fertilization membranes are not properly formed or are destroyed, and for eggs from which membranes have been removed, and which have been centrifuged at any stage after fertilization. This applies to both whole eggs and fragments. No study has been made of the later development of individual eggs or egg fragments, but observation of experimental lots shows that failure to develop depends chiefly on the falling apart of the cells due to the lack of an ectoplasmic layer and that both large (from whole eggs) and small (from fragments) plutei occur in lots centrifuged at any stage after insemination.

Summary

1. The ectoplasmic layer is *added on* to the surface of the egg of *Arbacia pustulosa* on fertilization.

2. The ectoplasmic layer can be thrown off the fertilized eggs of *Parechinus microtuberculatus, Paracentrotus lividus, Sphærechinus granularis, Arbacia pustulosa,* and *A. punctulata* by centrifugal force as a ring or crescent which lies in the perivitelline space.

3. The ring is not formed in absence of calcium, but the dissolved ectoplasmic material is precipitated when the eggs are returned to sea water as refringent spherules in the perivitelline space.

4. The ectoplasmic layer is reformed on eggs with fertilization membranes and the eggs develop normally.

5. Soon after insemination (1½–6 minutes), all the species studied break into many very small pieces; during the monaster stage (6–30 minutes), they form long streamers; later, elongate dumb-bells. The fertilized eggs of *Parechinus* and *Paracentrotus* break more readily at all stages than unfertilized eggs, those of *Sphærechinus* less readily; the fertilized eggs of all species stratify less rapidly than the unfertilized.

6. The female pronucleus is driven by centrifugal force to the light pole and the male pronucleus to the heavy pole of the elongate eggs.

7. The male pronucleus may become much larger than the female before fusion; the size of the male nucleus depends on the time before union and the density of the surrounding protoplasm.

8. An egg may be broken into two fragments, one containing the female and the other the male nucleus. Usually the former forms a monaster and does not develop, the latter an amphiaster followed by normal cleavage. In two lots of *Paracentrotus* eggs, the reverse took place; the division center was associated with the female nucleus and this fragment divided while the other fragment with the male nucleus did not divide.

9. The spindle is thrown to the light pole and cleavage usually comes in through its equator, perpendicular to the stratification. If centrifuged just before cleavage, the cleavage plane may come in parallel with the stratification and in no relation to the new position of the spindle.

10. Many whole eggs centrifuged after fertilization develop normally and also many fragments thus obtained. Failure to develop is due to the lack of an ectoplasmic layer which causes the cells to fall apart, or to the absence of one or both nuclei.

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Vol. LXVI, No. 3 June, 1934

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

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ELECTROKINETIC STUDIES OF MARINE OVA[1]

II. Cumingia Tellinoides, Asterias Forbesii, Echinarachnius Parma, Nereis Limbata and Cerebratulus Lacteus

KATSUMA DAN

(From the Zoölogical Laboratory, University of Pennsylvania, and the Marine Biological Laboratory, Woods Hole, Mass.)

In this study, an attempt was made to compare the cataphoretic potential of five types of marine eggs with that already described for sea-urchin eggs. The eggs chosen were those of the clam *Cumingia,* the starfish *Asterias,* the sand dollar *Echinarachnius,* the polychæte worm *Nereis,* and the nemertine *Cerebratulus.*

The method of measurement was the same as that described in the preceding paper (Dan, 1933). However, in this study the greater size of these eggs (except *Cumingia*) made necessary a slight modification in the procedure. The difficulty is that when, according to standard procedure, one tries to focus on an egg which is in the layer one-fifth of the distance across the chamber, the periphery of the egg comes so close to the wall that the latter exerts an influence on the movement of the egg. Strictly speaking, the egg is not free from this effect under any circumstances, but in case it lies sufficiently far from the wall, the effect becomes negligible. Therefore, I focussed to the middle layer of the chamber and studied the eggs in that layer. The effect of the electro-endosmotic current can be computed, as was described in the previous paper, from the graph obtained for this particular chamber. Another modification is the use of a lower magnification. *Nereis* eggs are very much heavier than the other types studied, and they therefore fall with a greater speed. As for *Asterias, Echinarachnius* and *Cerebratulus* eggs, even though their speed is not so great, their size is much larger than sea-urchin eggs and it is more convenient to observe them under a lower magnification. As a result, the accuracy of the data obtained in this way is not as great as that obtained by using a higher magnification, but it is, to be sure, good enough to determine the correct order of magnitude. When a lower magnification is used, the values obtained happen to be much smaller than those for *Arbacia* and *Cumingia* eggs, for which a higher magnification was adopted. This is, however, not an error due to the adoption of the lower magnification, for when *Arbacia* eggs with jelly are studied under similar conditions with low magnification, a charge of — 33.0 millivolts is obtained, and this value differs only 1 millivolt in absolute magnitude from the value obtained previously.

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[1] This paper and the preceding one of this series constitute a thesis presented to the faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

Cumingia tellinoides

Unfertilized Eggs with Jelly.—Freshly collected animals, after being kept dry for a while, were transferred to Stender dishes, one animal in each dish. After several females had shed eggs, these eggs were put together and the potential was measured. Some of the results are given in Table I. The average value of 23 measurements is — 34.1 millivolts.

TABLE I

Surface charge of unfertilized Cumingia eggs with jelly. Dielectric constant is taken as 80; viscosity is corrected to 25° C. (see text).

Potential gradient	Horizontal shift in 2.5 seconds	Values corrected for endosmosis and viscosity	Horizontal shift per second	Zeta potential
volt/cm.	*micra*		*micra*	*mv.*
6.7	42.9	43.3	17.3	−34.0
	39.4	39.8	15.9	−31.1
	41.1	41.5	16.6	−32.6
	47.0	47.4	19.0	−37.4
	41.6	42.0	16.8	−33.1
	41.6	42.0	16.8	−33.1
	45.8	46.2	18.5	−36.4
				Av. −34.0

In Table I, the dielectric constant of sea water is assumed to be 80, *i.e.*, that of distilled water. There are no measurements of the dielectric constant of sea water because of technical difficulties resulting from its high conductivity. As for the viscosity, the data for distilled water are used [2] and the cataphoretic speed is corrected to 25° C. to facilitate the comparison of these figures with those published in the preceding paper (Dan, 1933). The effect of temperature on the zeta potential itself is considered to be negligible within the range of temperature variation in this experiment (20°–24° C.) (Compare Burton, 1906).

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[2] There is a measurement of viscosity of sea water by Krümmel and Ruppin (1905). If their figures are taken, the absolute magnitude of the zeta potential may become greater by 6–7 per cent. But, since the zeta potential itself can be only a relative measure in so far as our present knowledge goes, the data for distilled water are adopted in conformity with the previous work.

Unfertilized Eggs without Jelly.—Eggs were secured in the same way as was described; then they were strained through bolting silk in order to remove the jelly. The result of 169 measurements gives the value of — 28.8 millivolts, the standard error being ± 0.46.

Asterias forbesii

Unfertilized Eggs with Jelly.—Eggs were obtained by cutting ovaries out of the body of a female and allowing mature eggs to stream out freely into the sea water. After fifteen or twenty minutes, the measurement was started. Eighty-two such measurements give an average value of — 19.0 ± 1.04 millivolts.

It may be noticed that the standard error is much greater here due to the adoption of a lower magnification. However, this is due mostly to the fluctuation involved in the process of free-hand tracing, as was discussed in the previous paper. This is shown by the fact that when eggs are cytolyzed and their speed of fall is thereby reduced, the measurement becomes relatively more accurate and a lower figure for the standard error can again be obtained, as will be seen later.

Unfertilized Eggs without Jelly.—Eggs, secured by the method which was described in the preceding section, were shaken vigorously so that the jelly would be removed. After shaking, samples were taken and were examined in Chinese ink suspension to determine whether or not the removal of the jelly was complete. Ninety-nine measurements were made, giving — 19.9 ± 1.01 millivolts as the average value. This figure is practically identical with that of eggs with jelly. In *Arbacia* and *Cumingia,* as well as in *Echinarachnius* (see below), the jelly shows a much higher absolute cataphoretic potential than the eggs. In so far as the data accumulated thus far are concerned, the *Asterias* egg is the only one in which the jelly has the same charge as the egg surface.

Eggs Killed by Heat.—There have been several studies concerning the effect of death upon the surface charge of cells. As early as 1906, Cernovodeanu and Henri discovered that bacteria retained their original charge materially unchanged, even after death of the cells by heat. Russ (1909) observed a similar fact with tubercle bacilli in urine. More recently Winslow et al. (1923) confirmed the earlier finding by using a more elaborate technique. Beside these facts, Abramson (1931) reported that " ghosts " of hæmolysed blood cells travelled with the identical cataphoretic velocity as that of intact cells. These observations are of great interest, but since they are concerned with rather simple and more or less quiescent materials such as bacteria or blood cells, it is very important to discover how more active cells would behave under similar circumstances.

Abramson (1928) made an observation in this direction. In his study of polymorphonuclear leucocytes, he compared the potential of active cells with that of degenerating cells. The latter were spherical in shape and non-amœboid. However, no difference was found between the speed of active and degenerating cells. He also pointed out that if human white cells were kept on ice for two days, there was no change in the cataphoretic speed. With these facts in mind, the following experiment was tried on *Asterias* eggs.

Table II

Surface charge of Asterias eggs (without jelly) killed by subjecting them to 40° C. for 5 minutes. Dielectric constant is taken as 80; viscosity is corrected to 25° C.

Potential gradient	Horizontal shift in 2.5 seconds	Values corrected for endosmosis and viscosity	Horizontal shift per second	Zeta potential
volt/cm.	*micra*		*micra*	*mv.*
6.7	31.5	25.9	10.3	−20.2
	31.5	25.9	10.3	−20.2
	25.7	20.1	8.0	−15.7
	31.5	25.9	10.3	−20.2
	36.2	29.4	11.8	−23.0
	24.5	18.9	7.6	−14.9
	29.1	23.5	9.4	−18.4
	33.9	28.3	11.3	−22.1
	31.5	25.9	10.3	−20.2
				Av. −19.4

Eggs were obtained in the same manner as before and the jelly was shaken off. Then the eggs were subjected to 40° C. for 5 minutes. At the end of this period, all the eggs were completely cytolyzed, and the average diameter, which was 146.8 μ before the treatment, now measured 174.6 μ. The cytolysis was so complete that there was no chance for any eggs to survive the treatment. Some of the results are given in Table II. The final figure from 85 measurements is — 19.0 ± 0.49 millivolts. It is surprising that the value for eggs killed by heat is identical with that of normal living eggs, in spite of the fact that the diameter and even the appearance of the cell has been so much changed by cytolysis.

Echinarachnius parma

Eggs were removed from females by cutting the tests open and putting ripe ovaries in an ample amount of sea water. Eggs were washed carefully. From 23 measurements, the average value for the potential of eggs with jelly is found to be —31.6 millivolts. Occasionally a piece of jelly which had been detached from an egg cell was found. In such a case, even though the jelly itself is invisible because of its transparency, its presence can be detected by the pigment granules which are scattered through it. If a piece of this sort was selected and its cataphoretic speed measured, it was found to move with about the same speed as intact jelly still attached to the surface of the cell.

In this form it is rather hard to get eggs free from jelly, because of the delicacy of the egg cells, so that it was never certain whether or not the removal of jelly was complete. However, eggs which were, so far as could be determined, without jelly, moved about two-thirds as fast as those with jelly. This fact may indicate that the potential of the naked surfaces of eggs falls somewhere around —20.0 millivolts. In one experiment tissue cells were involved in the egg suspension, and these tissue cells showed a speed of the same order of magnitude as egg cells without jelly.

Nereis limbata (*Unfertilized Eggs*)

The material was collected in the evening and was sometimes used immediately. Often, however, it was experimented upon early the following morning. The body of a female was cut open, the eggs were washed carefully, and they were then quickly brought into the chamber. Because of the great speed of fall of these eggs, the measurements are not as accurate as the preceding ones. The mean value of 76 measurements is —9.7 millivolts.

Cerebratulus lacteus (*Unfertilized Eggs*)

Eggs were secured by cutting open the body of a female. Some eggs were irregular in shape immediately after being taken out of the body, but later they became more spherical. The experiments were performed only after this state was reached.

As is well known, the *Cerebratulus* egg is enclosed in a huge chorion. This chorionic membrane has a certain degree of rigidity, but it is not difficult to remove it if desired. The large space within the membrane seems to be filled with some sort of a viscous organic fluid, for when seen from the side, the egg cell is always suspended, whereas if it is taken out into the sea water, it sinks very rapidly. Therefore in this case we must take account of the fact that the system we are dealing with is a composite one, consisting of three component parts: chorionic membrane, chorionic fluid, and egg cell. This is a very important point to keep in mind.

Surface Charge of the Chorion.—First, a chorion containing an egg was put into the cataphoresis chamber and its behavior in the electric current was observed. The chorion of *Cerebratulus* has an ovoidal form. Its long axis measures about 300 μ and the short axis about 200 μ. Therefore, taking 250 μ as the average diameter, for the purpose of a rough calculation, the strength of the endosmotic current which is originated by the wall of the cataphoresis chamber and is acting on the chorion is calculated. If this is done, since the observed speed is the addition of the true cataphoretic speed and the shift caused by the electroendosmotic flow, the former can easily be known. As a result of this calculation, it was found that the speed of migration of the chorion toward the anode far exceeded that which might be attributed to the electroendosmotic current acting on the chorion. Thus it is certain that the surface of the chorion is negatively charged.

Migration of the Egg Cell within the Chorion.—During the course of various experiments, chorions were sometimes found which, for some reason, were attached to the wall of the chamber. In this case, the chorion was incapable of free movement, and the behavior of the egg cell suspended in the chorionic fluid could be studied. The striking thing which was found in these cases was the migration of the egg cell toward the cathode (it is thus apparently positive). The speed of this cathodic migration was very small and, so far, no reliable figure has been obtained. After the egg reached one end of the chorion, it became somewhat flattened against the membrane, while the membrane itself was made to bulge out under the pressure of the egg. If the current was reversed, the egg regained its spherical form, changed its direction of migration, and travelled across the chorionic space very slowly. When it came to the other end, the same flattening occurred. This could be repeated many times with the same egg by reversing the current. The accompanying camera lucida drawing (Fig. 1) illustrates the phenomenon above described. This sketch, however, does not represent the highest degree of flattening, for the breaking of the current to permit drawing allowed the egg to resume its spherical form.

Movement of the Egg Cell in Sea Water.—In other experiments, the chorion was removed by straining the egg through bolting silk. The potential on the naked surface of the egg cell was measured in the same way as was done in the case of *Asterias* and other eggs. The mean value of 16 readings was found to be — 1.4 millivolts. Unfortunately 16 measurements is a very small number. *Cerebratulus* material is very difficult to obtain at Woods Hole, and only one worm was available during the entire summer of 1932 and none in 1933. As a result of the small number of determinations and the relatively high standard error, the value arrived at is not sufficiently reliable to establish the negativity of the potential. A greater number of determinations might very well give a small positive value, which would explain the slow cathodic migration of the eggs mentioned above. At any rate, for the present, it can at least be said with certainty that the charge on *Cerebratulus* eggs is in the vicinity of zero.

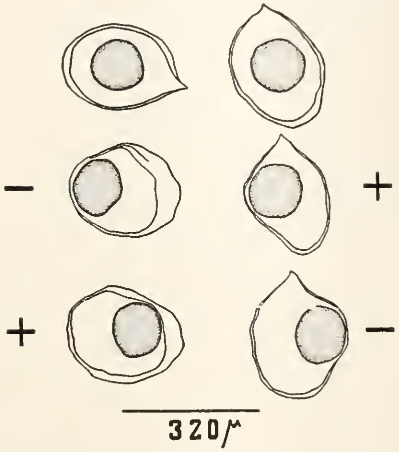

FIG. 1. Eggs of *Cerebratulus lacteus* within chorions. Each column represents the behavior of a single egg before and after the application of the electric current. Note the cathodic migration of the cells within their chorions.

In spite of the drawback in the measurement above stated, this egg indicates many interesting features. The first point to be noted is the peculiar fact that *Cerebratulus* eggs show so small a potential in such an alkaline medium as sea water (pH 8.2). Of course, there are several kinds of bacteria known to have a very low potential (in absolute magnitude) through a wide range of hydrogen ion concentration (Mudd and Joffe, 1933). Also there are several observations of the acquisition of zero or even a positive potential by bacteria in high pH values. The paper of Winslow et al. quoted above (1923) serves as one example of this phenomenon. Moreover, Vlès and Nouel (1922) and later Vlès (1924), in their experiments on agglutination of sea-urchin eggs at various pH values, found that there was a secondary agglutination point at an extremely high pH region. This might indicate, though indirectly, that sea-urchin eggs (*Paracentrotus lividus* Lk.) have a secondary isoelectric point in an extremely alkaline medium. At present, however, experimental data are too scanty to warrant speculation concerning the *Cerebratulus* egg. It is hoped that the investigation can be extended in this direction in the near future.

The second point of interest is the migration of these eggs within the chorion. This naturally leads to the question whether or not other eggs which are surrounded by transparent jelly behave in a similar way in an electric field. Therefore a qualitative observation was undertaken by using the eggs of *Echinarachnius, Asterias*, and *Arbacia*. The experiment consisted of sending an electric current through an India ink suspension containing eggs under cover slips. As soon as the circuit was made, the existence of a strong electroendosmotic current was revealed by the fact that all the ink particles on the cathodal side of the jelly were completely washed away from its vicinity. This is due to a current of water flowing through the jelly from the anodal side to the cathodal side, as is to be expected from the negative charge on the jelly noted above. However, in spite of this striking change in the surrounding medium, no shift was observed in the relative positions of the jelly and the egg cell. This is probably due to the difference in the physical nature of the chorionic fluid of *Cerebratulus* and that of the jelly layers of other eggs. On the other hand, Freundlich and Abramson (1927) discovered that red-blood cells migrate as fast cataphoretically in 1 per cent gelatine gel as in a sol of the same concentration. However, until the exact physical nature of the jelly is known, no conclusion can be drawn.

As for the flattening of the cell against the chorionic wall, Mazia (1933) reported a strikingly similar behavior in frog eggs (with jelly), and he also found that frog eggs migrated toward the cathode even after the jelly was removed by KCN. Another similar phenomenon was observed long ago by Carlgren (1900) in the parthenogonidia of *Volvox,* although the direction of the migration was opposite, namely toward the anode. Since I have not had the opportunity to study this material, I venture no opinion concerning it. At any rate, it is remarkable that a cell which is surrounded by a certain structure can migrate and flatten itself against the wall of its containing envelope.

Bungenberg de Jong (1932) has studied a somewhat similar phenomenon in non-living systems, and it is instructive to compare the analogous behavior of living and non-living systems.

Here I acknowledge my great indebtedness to Dr. L. V. Heilbrunn under whose direction this work was completed.

SUMMARY

1. By means of the method described in an earlier paper and a slight modification of it, the surface charge of five kinds of marine ova was measured.

2. *Cumingia* eggs with jelly show a charge of —34.1 millivolts (23 measurements); eggs without jelly show a charge of —28.8 ± 0.46 millivolts.

3. *Asterias* eggs with jelly have a charge of — 19.0 ± 1.04 millivolts; those without jelly — 19.9 ± 1.01 millivolts; and eggs without jelly which were killed by heat have a charge of — 19.0 ± 0.49 millivolts.

4. *Echinarachnius* eggs with jelly have a charge of —31.6 millivolts (23 measurements), and eggs without jelly seem to have a charge of about — 20.0 millivolts.

5. *Nereis* eggs, according to less accurate data, show a charge of about — 10.0 millivolts.

6. In the case of the *Cerebratulus* egg, the negative charge of the chorionic membrane was first ascertained; next the cathodic migration of the egg cell within the chorion was observed; and finally the surface charge of the cell in sea water was demonstrated to be in the vicinity of zero.

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THE HYALINE ZONE OF THE CENTRIFUGED EGG OF CUMINGIA

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The egg of the lamellibranch mollusk, *Cumingia tellinoides,* has been extensively used as material for studies with the centrifuge, beginning with the classical experiments of Morgan (1908*a,* 1908*b,* 1909, 1910). The results of these investigations indicated that the visible granular substances of the protoplasm, displaceable with centrifugal force, bore no necessary relation to the polarity, symmetry, or organ formation of the developing embryo. Since these embryological studies were made, *Cumingia* eggs have been centrifuged by Heilbrunn to study the changes in protoplasmic viscosity during maturation, fertilization, and cleavage (1920, 1921, 1925); to determine the effects of temperature on the viscosity of protoplasm (1924*a,* 1924*b*); and for measurements of the absolute viscosity of protoplasm (1926). E. N. Harvey has centrifuged these eggs to measure the tension at the surface (1931*a*), and as material for microscope-centrifuge studies (1931*b,* 1932). They were also centrifuged by Costello (1932), to determine the rôle of the granular constituents of the protoplasm in the surface precipitation reaction.

Morgan (1910) describes the appearance of the centrifuged unfertilized egg of *Cumingia* as follows: " When the egg is centrifuged the pigment and yolk are carried to the outer pole, the oil to the inner. Between the two lies a band of clear protoplasm." This description is based on sections as well as on the appearance of the living egg. Of the displaced granular constituents, it is the yolk alone, according to Morgan, which appears in the sections, the pigment and oil being dissolved by the reagents. The broad clear zone is figured as containing finely granular, stainable protoplasm. Morgan (1908*a,* 1908*b,* 1909) had previously described in less detail the three zones produced by centrifuging.

Morgan (1927, p. 501) has amplified the above description as follows: " After centrifuging, the egg shows four zones, similar to those in centrifuged eggs of the sea-urchin. There is (1) a large oil cap; (2) a broad, clear zone; (3) a small yolk field; and (4) a pink, pigmented region opposite to the oil cap."

All other workers have described but three zones. Heilbrunn (1920) says: "To one pole pass the presumably lighter oil globules, to the opposite pole the heavier pigment." Harvey (1931*b*) says: ". . . the oil is stratified in two-thirds the time necessary to stratify the yolk." These workers observed only living material.

The question naturally arises as to whether there are three zones and two types of granules in the centrifuged egg of *Cumingia*, or four zones and three types of granules (assuming the hyaline zone in both cases to be non-granular). It is quite evident that Harvey and Heilbrunn have observed but one type of granule at the centrifugal pole of the egg, and that in their descriptions yolk and pigment are synonymous. It is well known that the pigmentation of these granules may vary from deep pink to an almost colorless condition in different lots of eggs.

Unfortunately Morgan does not give the centrifugal force developed by the Bausch and Lomb hand centrifuge nor by the water centrifuge which he used in his experiments. From his figures, however, we may be certain that the forces used were small. Heilbrunn (1924*a*) used a force of 311 times gravity and stratified the eggs in 90 seconds or less. Heilbrunn (1925, 1926) used a force of 4,968 times gravity, and stratified the eggs in 5 seconds or less. Harvey (1931*a*) used a force of 6,595 times gravity to pull the eggs apart.

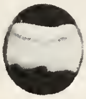
I have been interested in the application of great centrifugal forces for long periods of time. If the eggs are centrifuged for 10 to 20 minutes with a force of about 6,000 times gravity, in hæmatocrit tubes containing a centrifugal layer of isotonic sucrose, a stratification of the egg is produced markedly different from that described above. (See Photographs 1–10). There are visible in the living centrifuged egg the following zones: (1) an oil cap; (2) a clear hyaline zone containing no visible granules; (3) a broad zone of very small colorless granules; and (4) a zone of pink, pigmented granules. Zones (2) and (3) have been produced by separating out the finely granular material from the hyaline protoplasm in the broad, clear zone described by Morgan. The spindle may lie at any angle with respect to the stratification. The spindle generally lies in the zone of small colorless granules in the more strongly centrifuged eggs, or at the border of the hyaline zone and the zone of small granules in the less strongly centrifuged eggs.

Eggs centrifuged at any time after fertilization before the second cleavage may be stratified by prolonged centrifuging to give these same zones, though the duration of time of centrifuging to produce a given end-point varies with the phase of mitosis (see Heilbrunn, 1921).

If the eggs are centrifuged for 30 seconds with a force of 6,000 times gravity, or for a correspondingly longer time at lower forces, only three zones are produced. These are: (1) the oil cap; (2) the broad, clear zone; and (3) the pigmented zone.

The increase in height of the true hyaline zone formed by the movement centrifugalwards of the granules of the broad clear zone may be expressed as a function of the centrifuging time (for a force of about 6,000 times gravity). (See Table I.)

1.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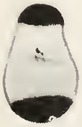
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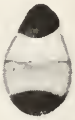
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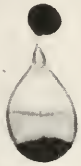
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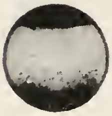
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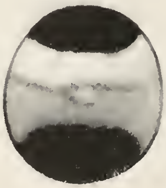
10.

EXPLANATION OF PHOTOGRAPHS

All photographs are of living unfertilized eggs, arranged with the oil cap uppermost in each case.

PHOTOGRAPHS 1 AND 2. Egg centrifuged 3 minutes, photographed 14 minutes later.

PHOTOGRAPHS 3 AND 4. Egg centrifuged 5 minutes, photographed 12 minutes later. Maturation spindle visible.

PHOTOGRAPH 5. Egg centrifuged 15 minutes, photographed 5 minutes later.

PHOTOGRAPH 6. Egg centrifuged 20 minutes, photographed 4 minutes later.

PHOTOGRAPH 7. Egg centrifuged 30 minutes, photographed 1 minute later.

PHOTOGRAPH 8. Same. The oil cap is pulling off as a spherical fragment.

PHOTOGRAPH 9. Egg centrifuged 1 minute, photographed 30 minutes later.

PHOTOGRAPH 10. Egg centrifuged 10 minutes, photographed 9 minutes later.

With prolonged centrifuging (30 minutes or longer), the oil cap separates as a sphere from the rest of the egg. (See Harvey, 1931*a*.)

In sections of centrifuged eggs fixed by the Champy-Kull technique, sectioned, and stained with Heidenhain's hæmatoxylin, the same four zones of stratification are found. The hyaline zone has coagulated to give a finely granular appearance. The small colorless granules remain, but do not stain intensely by this procedure. The oil globules are preserved, and appear as intensely black spheres at the centripetal pole of the egg. Since the term yolk has not been applied to any specific chemical substance in the eggs of marine invertebrates, the exact terminology to be applied to the pigmented granules seems of little importance.

Heilbrunn (1926) obtained a value of 0.043 poises for the absolute viscosity of *Cumingia* egg protoplasm. This value is based on the assumption that the pigmented granules were moving through hyaline or granule-free protoplasm. Actually, they were moving through the finely granular protoplasm of the broad clear zone. Therefore the absolute viscosity of the hyaline protoplasm would be somewhat lower than this figure. However, Harvey (1931*b*) gives a value for the average diameter of the pigment granules as 0.5 μ. My own measurements of granules in fixed preparations tend to verify this value, although measurements of such small particles are never very certain. Neglecting the Cunningham correction, this would give the suspension of the broad clear zone a viscosity of 0.118 instead of 0.043 poises. Several equations have been derived for estimating the viscosity of the dispersion medium when the viscosity of the suspension and the volume concentration of the suspended particles are known. For the egg of *Cumingia*, the volume of the zone occupied by the small colorless granules is about equal to the volume of the true hyaline zone. If this zone consisted of packed spherical granules of uniform diameter, they would occupy ap-

TABLE I

Centrifuging time in minutes	Height of hyaline zone in fractions of egg axis	Height of zone of small granules in fractions of egg axis
1/2	0	1/2
1	0	1/2
3	1/16	7/16
5	1/8	3/8
10	1/4	1/4
20	3/10 (egg pear-shaped)	3/10
30	2/5 (oil cap pulling off)	1/5

proximately 74 per cent of the total volume of this zone. However, there is probably considerable variation in granule size, and it is not certain how much packing occurs. As an approximation we may take the relative volume occupied by the granules in the broad clear zone as 37 per cent. The concentration of granules at which the viscosity is infinity is assumed to be 74 per cent, though this is probably too high. The equations for the viscosity of a suspension, with the values obtained for the viscosity of the hyaline protoplasm of the *Cumingia* egg, by the use of each, are listed below:

$$(1)\quad \eta_s = \eta\frac{1 + 0.5f}{(1 - f)^4} \qquad \text{(Einstein, from Kunitz, 1926)} \qquad \eta = 0.016$$

$$(2)\quad \eta_s = \eta(1 + 4.5f) \qquad \text{(Hatschek, 1910)} \qquad \eta = 0.044$$

$$(3)\quad \eta_s = \eta\frac{1}{1 - af} \qquad \text{(Hess, 1920)}$$

$$(3\text{a})\quad \eta_s = \eta\frac{1}{1 - \sqrt[3]{f}} \qquad \text{(Hatschek, 1911)} \qquad \eta = 0.033$$

$$(4)\quad \eta_s = \eta\frac{1}{(1 - f - 3/2f^{5/3})^{5/2}} \qquad \text{(Smoluchowski, 1916)} \qquad \eta = 0.008$$

$$(5)\quad \eta_s = \eta\frac{1}{\left(1 - \dfrac{f}{f_\infty}\right)} \qquad \text{(Bingham and Durham, 1911)} \qquad \eta = 0.059$$

where η_s is the viscosity of the suspension (0.118), η is the viscosity of the dispersion medium, f is the ratio of the volume of suspended particles to the total volume of the suspension (37 per cent), f_∞ is the value of f at which viscosity becomes infinite (74 per cent), and a is a constant.

As Kunitz points out, equation (1) is purely empirical. Equation (2) may be applied in cases where f is large. Equation (3), involving the constant a, cannot be applied except by assuming

$$a = \frac{1}{\sqrt[3]{f^2}}\,(= 1.94),$$

which makes this equation identical with one previously derived by Hatschek (3 *a*) for very concentrated suspensions ($>$ 50 per cent) of deformable particles. Hess has applied this equation to suspensions of both low and high concentration. Equations (4) and (5) are also empirical. It is not possible to state at present which of these equations applies best to this particular case, so the values calculated are indicative only of the order of magnitude of the viscosity of the hyaline proto-

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plasm. Obviously, any value lower than the viscosity of water (0.01 poises) is an impossible figure.

Completely stratified *Cumingia* eggs were crushed in the manner described by Costello (1932) and the presence or absence of a surface precipitation reaction observed for the different zones of stratification. This was done to determine whether or not the small colorless granules are directly involved in the surface precipitation reaction, as Heilbrunn has previously shown for the pigment granules of the *Arbacia* egg. It was usually possible to obtain a surface precipitation reaction from the heavy pole of the egg, if the egg were gently ruptured. From protoplasm extruded at the light pole, however, a definite membrane was never obtained. Whether this is due to the greater fluidity of the protoplasm at this pole, or due to the absence of a particular type of granule, could not be determined. Since the granules of the zone of small colorless granules are about at the limits of microscopical vision (0.2 μ or less in diameter), it was not possible to determine whether or not they disintegrated during the formation of the surface precipitation membrane at the heavy pole of the egg.

Considering the results outlined above, Morgan's (1910) analysis of the relation between organ-forming substances and embryonic localization in the egg of *Cumingia* is somewhat incomplete, since it does not preclude the possibility of almost normal distribution of the small colorless granules of the broad clear zone to all of the early blastomeres. (The small colorless granules are of such a size that they could occupy the interspaces between the packed pigment granules or oil in the eggs centrifuged with the forces used by Morgan.) However, considering the results of the centrifuge experiments on other eggs, it seems improbable that the small colorless granules are any more morphogenic than the other types of granules. (But compare the results of Conklin, 1931, obtained by prolonged centrifuging of the egg of *Styela*.)

SUMMARY

1. The zones of stratification of the centrifuged egg of *Cumingia* have been redescribed.

2. The relations of the presence of granules in the "broad clear zone" to previous work on (*a*) absolute protoplasmic viscosity, (*b*) the surface precipitation reaction, and (*c*) embryonic localization have been discussed.

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THE EFFECT OF ANESTHETICS ON THE SURFACE PRECIPITATION REACTION

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Living substance is unique in its capacity to respond to sudden changes in its environment. This innate irritability may, however, be lost temporarily under the influence of various anesthetics. In any general interpretation of irritability, the phenomenon of anesthesia must be carefully considered, and, indeed, if we could explain anesthesia we would be well on our way toward an interpretation of what actually happens when protoplasm is aroused to activity. It is not surprising, therefore, that so many authors have attempted to solve the riddle of anesthesia. Much of this work is summarized in Winterstein's excellent monograph (1926). A more recent review is that of Henderson (1930). The paper of Bancroft and Richter (1931) may also be consulted.

Some years ago, I began to gather data in favor of the view that stimulating agents cause a gelation of the protoplasm and that the primary action of anesthetics was to prevent such a gelation. This point of view was stressed in a monograph published in 1928. Facts were presented to show that stimulation does actually cause a very sharp increase in protoplasmic viscosity and that such general stimulating agents as the electric current or ultraviolet radiation do indeed stiffen the protoplasmic fluid. An attempt was also made to interpret the gelation involved in stimulation. Response was thought to be due to a type of clotting, the mechanism of which was essentially similar to that involved in the injury reaction which occurs when a cell is torn or broken. As the protoplasm begins to emerge from such a torn cell, a peculiar and distinctive type of precipitation reaction occurs. This I call the surface precipitation reaction, or s.p.r. A new film or membrane appears, and this membrane formation at the border of the exuding droplet may be accompanied by the formation of numerous new films within the droplet. As a result, many small vacuoles frequently appear, and this vacuolization may extend throughout the entire cell. Such a complete vacuolization usually leads to death, which is frequently the result of overstimulation. The gelation which occurs after normal stimulation is thought to involve a reaction of the same general nature as the surface precipitation reaction, and it may be interpreted in terms of this reaction. In the present discussion, I shall limit myself to the simple concept outlined above, although the actual events may be much more complicated (compare Heilbrunn and Daugherty, 1933).

If, then, stimulation causes clotting of protoplasm, and if, moreover, this clotting is essentially an internal surface precipitation reaction, then it should follow, first, that anesthetics prevent clotting or gelation in the cell interior, and, second, that they should have an inhibiting effect on the s.p.r. In earlier work, I was able to show that ether and other fat solvents do actually exert a liquefying action on protoplasm, and do prevent the gelation which appears on stimulation (for details consult Heilbrunn, 1928). This much is in accord with theory. But, if the gelation which accompanies stimulation is fundamentally the same type of reaction as the s.p.r., then it should be possible to demonstrate an effect of fat solvents on the s.p.r. as it occurs when a cell is torn or broken.

Earlier attempts in this direction met with failure. When sea-urchin eggs were torn or broken in the presence of ether dissolved in anesthetic concentration in the surrounding sea water, the normal type of precipitation reaction occurred, and there was not the slightest evidence of any retarding effect of the ether. Various fat-solvent anesthetics were tried, and in no case was the s.p.r. retarded or prevented in their presence. Observations of this sort constituted a serious stumbling block to the point of view which I have been advocating.

It must be remembered, however, that when a sea-urchin egg is torn or broken in sea water, the surface precipitation reaction occurs in the presence of an abundance of free calcium. On the other hand, if an s.p.r. were to occur within the cell, the reaction would presumably take place in the presence of a very small amount of calcium, for there is good reason to believe that within the cell most of the calcium is bound chemically. In view of the fact that calcium plays such an essential rôle in the s.p.r., this is an important difference.

During the past summer, in the course of some experiments with the magnesium ion, I found that ether prevented the surface precipitation reaction which this ion may produce. It will be shown later that magnesium acts like calcium, except that its effect is much less potent. Thus, a dilute solution of calcium acts like a much stronger solution of magnesium. It was decided, therefore, to test the effect of ether on the s.p.r. in the presence of a very low concentration of the calcium ion. Under these conditions, it was indeed found that ether does prevent the s.p.r. Some of these observations will now be presented in detail.

Action of Ether on the S.P.R. in Arbacia

Only the simplest type of experiment was performed. In my original description of the s.p.r. in the *Arbacia* egg (Heilbrunn, 1927), it was shown that a trace of calcium was sufficient for the reaction. Thus if eggs are placed in isotonic sodium chloride solution and crushed, there is enough calcium carried over with the eggs to permit a reaction. If, however, eggs are washed once or twice in isotonic sodium chloride solution and then crushed, no reaction occurs.

Experiments were performed with solutions which contained 1000 parts of an isotonic sodium chloride solution (0.53 M) to one part of an isotonic calcium chloride solution (0.3 M). One drop of egg suspension was placed in 20 cc. of such a solution, and then after a minute a drop of the eggs was pipetted into a second dish also containing 20 cc. of the same solution. The eggs were then placed on a slide under a cover and crushed. The crushing was usually accomplished by sucking out fluid from the side of the cover slip with a piece of filter paper. When the pressure becomes great enough, the eggs break, the protoplasm streams out, and the small exuding droplet forms a membrane around itself. Pigment granules in the extraovate gradually disappear from view. There is thus a typical s.p.r. A peculiar phenomenon then follows in most of the eggs which are crushed in these solutions containing little calcium. Membrane elevation similar to that produced by the sperm in the fertilization reaction now occurs in that part of the egg which has remained intact. Starting from the rim of the extraovate, that is to say, from its junction with the uninjured protoplasm, the vitelline membrane slowly starts to lift away from the cytoplasm of the egg. This membrane elevation proceeds around the egg surface until the entire uninjured portion of the egg has a perfect fertilization membrane. It is believed that this observation of membrane elevation following injury is of real importance for the theory of membrane elevation, but I shall not discuss it further in this paper, except to point out that the phenomenon occurs only when the calcium concentration is low. In sea water there is no evidence of it.

If eggs are immersed as before in 1000 parts by volume of isotonic sodium chloride solution plus 1 part of isotonic calcium chloride solution, with now the addition of 2 or 3 cc. of ether per 100 cc. of solution, a different result is obtained. To prevent the evaporation of ether, the eggs are placed in glass-stoppered weighing bottles, but otherwise the treatment is the same. When the etherized eggs are crushed under a cover slip, no surface precipitation reaction occurs, and there is no subsequent membrane elevation. Usually the pigment granules emerging from the crushed eggs remain intact and visible. The ether has thus interfered with the surface precipitation reaction, and has prevented both breakdown of pigment granules and formation of a new film or precipitation membrane. (Membrane elevation is also inhibited.)

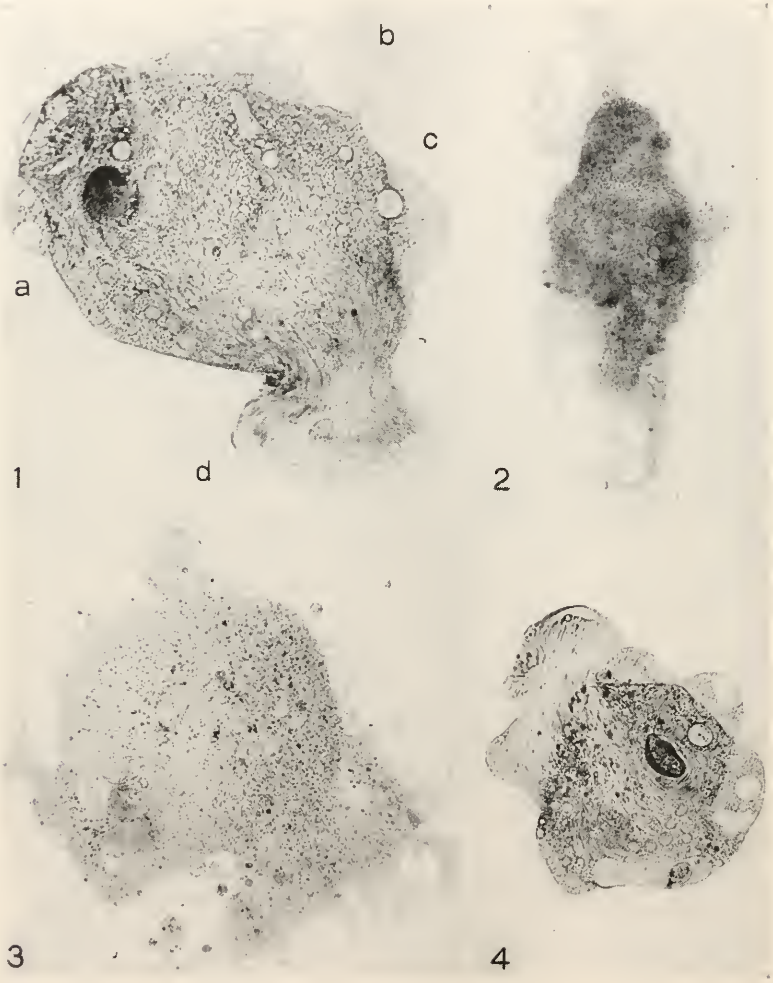
Ether can thus prevent the s.p.r. in the *Arbacia* egg, but this antagonism occurs only when the calcium concentration is low. If the calcium concentration is doubled, that is to say, if the eggs are crushed in 500 parts of isotonic sodium chloride solution plus 1 part of isotonic calcium chloride solution, the effect of the ether is not so noticeable, and with slightly higher concentration of calcium, it disappears completely.

The action of ether on the s.p.r. may be demonstrated very simply by comparing the reaction in an isotonic sodium chloride solution with that which occurs in a similar solution containing 2 per cent ether (by volume). When a drop of egg suspension is added to 20 cc. of an isotonic sodium chloride solution, and the eggs are crushed, there is a clear-cut reaction involving both breakdown of pigment granules and formation of a new surface film. On the other hand, in the presence of ether, the reaction is inhibited. In performing this experiment, eggs should be tested within a few minutes after immersion into the solutions, for with increasing length of exposure to isotonic sodium chloride, the surface precipitation reaction becomes less distinct and the pigment granules tend to remain intact on escaping into the sodium chloride solution. A similar time effect can also be noted in the case of magnesium solutions.

Action of Ether on the S.P.R. in Stentor

The action of ether in suppressing the s.p.r. may be very clearly demonstrated for the protozoön *Stentor*. This form is ideal for a study of the reaction and when the cell membrane of the stentor is broken, a beautiful s.p.r. occurs. Through the kindness of Dr. Hetherington, I was able to secure an excellent culture of *Stentor cœruleus*, and numerous experiments were performed with it. Later other cultures were obtained in Philadelphia. In these the animals were reared in a dilute wheat infusion in pond water. Similar results were obtained with them.

When *Stentor* is placed on a slide, and a cover slip is pressed down over it so that the protoplasm is forced out through a rupture of the body wall, the emerging droplet immediately forms a membrane about itself. I know of no protozoön which gives a clearer reaction. Figure 1 is an unretouched photograph which shows the phenomenon clearly. Several droplets with their membranes appear at *a, b, c.* There is evidently a sharp border between the protoplasm which has been pressed out of the cell and the surrounding medium. At *d* the protoplasm is moving out of the cell, and a membrane is just being formed. The s.p.r. in *Stentor* is also shown in Fig. 4, in which the background has been blocked out for purposes of contrast.



EXPLANATION OF PLATE I

FIGS. 1 AND 4. Surface precipitation reaction in *Stentor*.

FIGS. 2 AND 3. Absence of surface precipitation reaction in the presence of 1 per cent ether.

The magnification is × 80 for Figs. 1 and 3, and × 50 for Figs. 2 and 4.

If, now, stentors are immersed in 1 per cent ether in tap water (Woods Hole or Philadelphia) and crushed in the same manner as before, no s.p.r. occurs, and the protoplasmic granules stream through the solution. This is clearly shown in Figs. 2 and 3, unretouched photographs. In order to make certain that the effect is due to the ether and not to the tap water, control experiments were performed in which animals were crushed in tap water alone. In such tests, a perfect s.p.r. was obtained. It is evident, therefore, that in *Stentor,* as well as in *Arbacia,* ether exerts an inhibiting effect on the s.p.r. A concentration of ½ per cent ether is not as effective as 1 per cent ether in stopping the reaction. In 2 per cent ether, no typical s.p.r. occurs, but the protoplasmic granules from crushed specimens do not scatter through the solution as is the case when 1 per cent ether is used.

Results similar to those obtained with ether were noted when *Stentor* was immersed in 1 per cent butyl alcohol and crushed. Ethyl urethane, amyl alcohol, and acetone were also effective in preventing the s.p.r.

The results obtained indicate that ether (and other fat-solvent anesthetics) can exert an inhibiting effect on the s.p.r. This effect is more readily observed in *Stentor,* for it may be noted when ether is added to the solutions in which *Stentor* lives. On the other hand, in the case of sea-urchin eggs, in order to show the effect of ether, one must study its action in solutions which contain only a very small amount of calcium.

Moreover, in the case of sea-urchin eggs, when ether in relatively high concentration (3–4 per cent) is added to the sea water surrounding them, the eggs undergo a vacuolization reaction which is thought to be essentially an internal surface precipitation reaction. Thus, although under some conditions ether may inhibit the s.p.r., under other conditions it may actually initiate the reaction. The latter effect is believed to be due to the fact that ether releases free calcium into the egg interior. This free calcium then initiates the reaction even though ether is present.

It thus appears that ether (and presumably fat solvents generally) can both favor an s.p.r. and prevent it. We thus have a formal explanation of the queer fact that fat-solvent anesthetics are sometimes stimulants. In the case of the *Arbacia* egg, the gelating or stimulating effect of ether preponderates at higher concentrations of the fat solvent.

Relation of the Magnesium Ion to the S.P.R.

Most theories of anesthesia find the fact of magnesium anesthesia a stumbling block. They have no way of explaining it. Magnesium, it is true, may lower permeability, but its chief antagonist is calcium, which has a similar effect on permeability. This difficulty has bothered some of the adherents of the permeability doctrine, so much so that

Höber (1926) has been led to state that magnesium anesthesia is not a true anesthesia; for, says he, true anesthetics are all surface-active, they penetrate cells and paralyze all their functions reversibly, at least more or less, whereas magnesium acts primarily at cell junctions or synapses. In view of the fact that magnesium salts are perhaps the most widely used anesthetics for marine invertebrates, and have an anesthetic action on widely divergent organisms from amœba (Heilbrunn, 1932) to man, it is not so easy to dismiss them as anesthetics. Perhaps, after all, it is a question of definition. Personally, I doubt if magnesium is less of an anesthetic than ether.

How then shall we explain the action of the magnesium ion? In a recent note (Heilbrunn, 1932; see also Heilbrunn and Daugherty, 1932) it was shown that magnesium tends to liquefy the plasmagel of the amœba. This fact fits in well enough with the general theory of anesthesia outlined above, and it is also in accord with other evidence as yet unpublished which indicates that various types of anesthetics all liquefy the plasmagel of *Amœba proteus.* But there is another side to our theory, for stimulation is thought to involve a reaction related to the s.p.r. What effect then does the magnesium ion have on the s.p.r. and is it possible to relate such an effect to the known facts of anesthesia?

In my 1927 paper, the relation of magnesium to the s.p.r. was considered briefly and not very correctly. It was stated that magnesium was not a good substitute for calcium, and that when an s.p.r. occurred in magnesium solutions, it was due to a secondary process. As a matter of fact, magnesium may be substituted for calcium. Indeed, an excellent s.p.r. occurs in isotonic solutions of magnesium salts.

When *Arbacia* eggs are immersed in a solution of 0.3 M magnesium chloride, and are then crushed after a minute or two, there is a clear-cut reaction at the surface of the exuding droplet of protoplasmic fluid and an excellent membrane is formed. Moreover, the pigment granules break down, or at least some of them do. As a matter of fact, the behavior of the pigment granules in these eggs is very interesting. Typically, when a droplet of protoplasmic fluid begins to emerge from the torn surface of the egg, the pigment granules are at first intact in the exuded mass. Breakdown of granules then begins in a very limited region. The broken egg is dumbbell-shaped, consisting of a large sphere which is the original egg cell, and a smaller sphere, the exuded droplet or extraovate. Between the two there is a relatively narrow neck of material. It is in this neck, the original cortex of the egg in the injured area, that pigment-granule breakdown occurs. From this initial point of pigment-granule breakdown, a wave of reaction spreads in both directions into and across the original egg cell on the one side, and out into the exuded droplet on the other. The phenomenon is very striking. As the pigment granules break down at any one point, they release a bright red color, and vacuoles come into view. At the next instant, neighboring granules go through the same process and the reaction is repeated across the cell. Often the wave of reaction goes completely across the original cell and the extraovate, so that all parts of the dumbbell are affected. The speed of the wave can readily be measured. Usually at room temperatures it takes approximately 15 seconds for the wave to pass to the farthest end of the cell. There is more or less variation in speed with different degrees of injury. Even so, fairly consistent values may be obtained. Thus, six tests showed 15, 14, 18, 14, 21, 15, 18 seconds. When eggs are crushed in isotonic calcium chloride solution, there is a similar wave of reaction across the cell. In this case, however, it appears to be more rapid, and the egg is usually traversed in about 5 seconds.[1] In calcium chloride solutions, breakdown cannot be seen to begin at the neck of the dumbbell, but occurs almost instantly in all of the exuded droplet. In solutions of magnesium chloride, the wave of reaction is often halted before it passes across the egg or before it crosses the extraovate. Thus, in eggs crushed in magnesium chloride solutions, one often sees intact pigment granules in the extraovate. Once the reaction wave has been halted, the pigment granules beyond its path seem to acquire a resistance to breakdown. This applies both to the granules within the egg proper and those in the extraovate.

When the s.p.r. is observed in sea water, the pigment granules in the extraovate all break down and there is also a wave of breakdown which passes across a fraction of the original egg. Usually the wave across the egg itself is halted before it proceeds very far.[2] If, now, the egg be crushed a second time, those pigment granules which resisted breakdown after the first injury lose their pigment and disappear. Eggs in magnesium chloride solutions behave differently. If they are crushed once, and if the wave of pigment-granule breakdown fails to include the whole egg, those granules beyond the path of the wave become resistant to breakdown. When the egg in magnesium solution is crushed a second time, the intact pigment granules do not break down, even though they float free in the surrounding medium. It is thus apparent that under the conditions described above, the pigment granules have acquired an immunity to breakdown.

It is possible that in this observation lies a clue to the nature of the anesthetic action of the magnesium ion. We can conceive of living protoplasm as being always in a state of unstable equilibrium between those factors which favor an s.p.r. and those which inhibit or reverse it. Then, once an s.p.r. occurs, it must, to some extent at least, be reversed before the protoplasm can become irritable again, so that normal stimulation in itself involves as a necessary sequence the reversal of the reaction involved in stimulation. It is possible that anesthetics sometimes act by causing a mild stimulation followed by an immediate reversal. The stimulation may be completely hidden so that the observer sees only the reversal, and there is apparently only anesthesia. The fact that in magnesium solutions granule breakdown, which may be regarded as a primary part of the stimulatory gelation, may be inhibited, opens the possibility of explaining the action of magnesium salts in terms of the theories outlined above. It is at least clear that under certain conditions, magnesium may be regarded as tending to inhibit the s.p.r.

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[1] These values for speed of reaction wave across the cell are for eggs crushed within a few minutes after immersion into the solutions. Following longer times of immersion, the waves move more slowly.

[2] This stoppage of the wave is apparently due to the presence of sodium ion.

However, this is not the only way in which the anesthetic action of magnesium may be interpreted in terms of the s.p.r. It has already been noted that both calcium and magnesium can initiate the s.p.r.[3] Calcium is effective in extremely low concentration. In experiments cited above. the s.p.r. was found to occur in solutions in which one part of isotonic calcium chloride solution was mixed with a thousand parts of isotonic sodium chloride solution. Actually, one part of calcium solution in two thousand parts of sodium solution is sufficient. Not so with magnesium; it must be present in much higher concentration. Experiments were performed in which eggs were placed in various mixtures of isotonic sodium chloride (0.53 M) and isotonic magnesium chloride (0.3 M). In such experiments it is essential to wash the eggs in one dish containing a given mixture and then transfer them to a second. In each transfer a single drop of egg suspension was carried over to 20 cc. of the solution. This procedure is necessary in order to wash the eggs relatively free from calcium ion. Apparently there is some variation between different lots of eggs, nor is it always easy to state precisely just when an s.p.r. occurs and when it is absent, for there is, of course, a gradual transition between a complete reaction and none at all. It is certain, however, that 1 part of magnesium solution to 39 parts of sodium solution is not sufficient to cause an s.p.r. when the eggs are crushed in this mixture. Neither is 1 part magnesium solution to 19 parts sodium solution. In mixtures of 1 part magnesium solution to 9 parts sodium solution, the reaction may or may not occur. This is a boundary concentration and mixtures which contain appreciably more than 1 part of isotonic magnesium solution in 10 parts of the total solution are certain to give the reaction. Thus, the magnesium ion is relatively weak in comparison with the calcium ion; the latter is at least a hundred times more powerful in promoting the s.p.r.

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[3] It is possible that only calcium is directly involved in the reaction and that magnesium acts by releasing calcium from some chemical union in the protoplasm.

These observations suggest a second type of explanation for the anesthetic action of the magnesium ion. It is reasonable to assume that in living systems the magnesium ion can replace the calcium ion. It has been assumed (Heilbrunn, 1928; Heilbrunn and Daugherty, 1933) that the stimulating gelation within cells is due to a release of calcium ion. Suppose now that a cell anesthetized with magnesium has its calcium compounds to some extent replaced with magnesium. If the replacement were complete, stimulation would, according to our theory, require the release of at least 100 times as much free ion if this were magnesium instead of calcium. Thus the magnesium-treated cell becomes relatively resistant to stimulation.

General Discussion

When a living cell is aroused to activity, changes in its protoplasm must occur. These changes are almost certainly not confined to the osmotic membrane of the cell, and the main mass of the living substance is doubtless affected. Protoplasm is a colloidal material and we are therefore faced with a problem in colloid chemistry.

In interpreting stimulation and the action of anesthetics which prevent it, many authors have reasoned from the known effects of stimulating agents and anesthetics on inanimate colloids. The primary information concerning the colloid chemistry of protoplasm has clearly shown the inadequacy of such reasoning. Protoplasm is vastly different from any known inanimate colloid. It is, therefore, essential to study the effects of stimulants and anesthetics on protoplasm itself. Unfortunately, many of the cells which give the most interesting responses when stimulated can not readily be studied from a colloid chemical standpoint. If we are to make progress in this field, the only sure method at our disposal at the present time is to study the effects of stimulants and anesthetics on those cells whose protoplasm lends itself to colloid chemical study. Thus we can determine the effect of ether on various types of protoplasm, and the knowledge so gained is of more importance to the theory of anesthesia than any information derived from non-living materials.

All protoplasm is very sensitive to physical treatment and chemical reagents. It is easily injured, and any excessive stimulation leads to injury or death. Indeed, it is probable that stimulation is equivalent to slight injury. Almost all agents which stimulate or injure protoplasm cause a peculiar reaction in it. The fluid protoplasm becomes stiffer and this gelation when carried to the point of injury is seen to involve a characteristic vacuolization reaction. Ultraviolet radiation, X-rays, the electric current, sudden pressure, heat, all produce this reaction. How shall we interpret it?

In earlier studies, I have shown that the vacuolization reaction is the same reaction as that which occurs when a cell is torn or broken, that is to say, it is essentially an s.p.r. In many cells the s.p.r. is very easy to study, and one can readily determine the effect of various reagents upon it. But it should be clearly understood that the s.p.r. is an extraordinarily complex reaction or series of reactions. In many respects it resembles blood coagulation. Probably the fundamental reaction of living cells, their power to clot when torn or broken, has been taken over by the blood of many animals, so that there is a deep underlying relationship. It may be remembered that in blood clotting, cell or tissue extracts play important rôles.

In spite of the numerous studies of blood clotting, the process is still very incompletely understood. Only very few authors have studied the clotting of the protoplasmic fluid, so that our knowledge in this field is only at the beginning.

If the attempt is made to develop a logical theory of stimulation based on our present knowledge of the colloidal chemistry of protoplasm, it should be possible in such a theory to account for the action of anesthetics. Apparently protoplasm is in a delicate state of balance between the forces which favor clotting or gelation and those which inhibit or prevent it. With minor reservations, it may be said that stimulation implies a gelation of the main mass of the protoplasm, and anesthesia a solation. If the gelation produced by stimulating agents is essentially an s.p.r., then it should be possible to show that anesthetics prevent or retard the s.p.r. That has been the purpose of the present contribution. For the first time, I have been able to show that fat-solvent anesthetics do actually prevent the s.p.r. in certain types of living material. Moreover, the relation of the magnesium ion to the s.p.r. indicates two possible ways in which this anesthetic ion may retard or prevent the reaction. It has thus been possible to add an additional step in the colloid chemical interpretation of stimulation and anesthesia.

SUMMARY

1. In the presence of low concentrations of calcium, ether inhibits the surface precipitation reaction in *Arbacia* egg cells.

2. Ether and other fat solvents also inhibit the surface precipitation reaction in *Stentor* protoplasm.

3. The magnesium ion can cause a surface precipitation reaction in *Arbacia* eggs, but it is far less potent than calcium. The course of the reaction in magnesium solutions is described.

4. There are two ways in which the presence of magnesium might act to prevent the surface precipitation reaction.

5. The relation of these facts to the colloid chemical interpretation of anesthesia is discussed.

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H-ION CONCENTRATION AS A FACTOR IN THE TOXICITY OF AMINES FOR AMŒBA PROTEUS

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INTRODUCTION

Although it is generally admitted that changes in the pH values of solutions of weak bases influence their penetration rate, the question as to whether free bases or their dissociated ions pass through cell membranes has been a matter of controversy since the time of Overton (1900). In 1921, Crane found that the magnitude of the toxic effect of alkaloids on *Paramecium* varies directly with changes in H-ion concentration, and on comparing the dissociation constants of the bases used, recognized a direct relation between them and the results obtained. She concluded that the undissociated base is responsible for the toxic action. Irwin's investigations (1926, 1930–1) on the penetration of dyes into *Nitella* have added support to this view. Moreover, the facts which many experimental methods have yielded in regard to the nature of protoplasmic boundaries, led Osterhout, in 1931, to state that "electrolytes must travel chiefly in molecular form in passing through this layer."

On the other hand, Brooks, although she found that certain basic dyes (1929) penetrate more rapidly from an alkaline medium, and acid dyes (1932) more rapidly from media of lower pH, was unable to fit the curve in which rate of penetration was plotted against pH to the dissociation curve of the dye. She therefore expresses the guarded opinion that more than one factor is important in determining penetration rate. In view of these facts, we have thought that an examination of the influence of pH changes on the penetration of an extended series of the simplest nitrogen bases, the amines, would be of interest, especially as a very considerable proportion of the physiologically active compounds belong to this class of substance.

MATERIALS AND METHODS

The amines used were the "Eastman" grade, marketed by the Eastman Kodak Company. The solutions were made up with water twice glass-distilled, from a stock kept in alkali-free glass containers.

Amœbæ, all of the same species, *Amœba proteus*, were found to be satisfactory organisms for the toxicity tests. For observing and counting them a binocular microscope equipped with 10 × oculars and 3.4 × objectives was used in conjunction with the lighting unit described by Chambers and Pollack (1927), and found by them to be satisfactory for the observation of intracellular color changes. The animals were handled by means of a mouth pipette drawn out into a long capillary with a bore sufficiently wide to prevent injury to the plasma-membranes of the cells, and a volume small enough to insure the transfer of but a minimal amount of fluid. Amœbæ for each experiment were picked up individually from a single culture, washed in a large quantity of glass-distilled water, and left over night in this medium. At least 550 amœbæ, selected from those actively crawling and apparently in excellent physiological condition, were subjected to the first washing. This number was reduced by selection to 500 during the transfer to the second (overnight) washing fluid, and again to 450 in seeding the immersion dishes the next morning.

The number of animals used in a given experiment was checked by counting them as they were picked up in the pipette and again after they had been introduced into the immersion dishes containing the amine solutions. The dishes were of the Syracuse type, the under surfaces of which were scored off into quadrants to facilitate counting. During the period of an experiment the dishes were covered with inverted watch glasses to minimize evaporation and prevent the entrance of dust.

Preliminary trials with 10 to 35 amœbæ showed that 30 in a single dish could be quickly and accurately counted without confusion. By a curious coincidence this is the number of animals which Trevan and Boock (1927) consider the mathematically optimum number for the determination of average lethal doses.

With each amine, trial determinations were made to ascertain approximately what concentrations would give definite toxicity effects within a twelve-hour period. The limitation of the experiments to this time interval insured a minimum of bacterial or mould infection and of evaporation, and at the same time gave results fully as satisfactory as those extending over a period of two or three days.

During the experiments the laboratory held a surprisingly constant temperature in the neighbourhood of 20° C.

Four dilutions of the buffered amine were employed at each pH, and the degrees of pH chosen were 6.4, 7.0, and 7.8, which cover the viability range of *A. proteus* without approaching the acid and alkaline death limits too closely. The pH of each stock solution was tested colorimetrically before the introduction of the amœbæ, and, at the end of each experiment various dishes, chosen at random, were examined to make certain that the buffer [1] had kept the cultures at a constant pH.

Criteria of Death

Since the data compiled on the toxicities of the amines are based entirely on mortality counts of amœbæ immersed in solutions of these substances, the necessity of establishing the death point of the amœbæ with the utmost possible degree of accuracy cannot be overestimated. Those criteria must, furthermore, be so definite as to assure their instant recognition, and thus insure identical counts by different observers. Many criteria which on *a priori* grounds might be expected to be of value in determining a death point were debarred for various reasons. For example, death of amœbæ, in the concentrations of amines used, was not accompanied by disintegration or dispersion of the organisms. They remained over a period of many hours as soft coagula. Though cessation of locomotion was quite uniformly a precursor of death, this in itself could not be used as a criterion, for motionless animals transferred to culture media often recuperated. The presence or absence of cyclosis in the internal protoplasm could not be accurately observed with the low power magnifications essential to rapid counting. Rounding of the cell, even over proportionately long periods of time, was found to be unreliable as an indication of death, inasmuch as rounded amœbæ, transferred to a normal culture medium, frequently recovered completely.

In animals immersed in amine solutions, however, there occurred a change in color from the normal gray appearance to a cloudy brown or tan, and such animals never formed pseudopodia, never showed cyclosis when examined under highest power, and never recovered on transfer to culture medium. This contrast between gray and cloudy brown animals could be detected under the low power binocular with ease and accuracy.[2] Color change in the animals was usually preceded by cessation of locomotion and by contraction or rounding. In dimethyl and trimethyl amines an initial strong contraction of the animal, accompanied by lifting of the pellicle into numerous petaloid blisters, was followed by swelling of the organism to a spheroid form, after which the color change followed gradually.

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[1] Sodium phosphate buffers (M/280) were used, the formulæ for which are given by Cohen and Clark in their Studies on Oxidation Reduction, Hygienic Laboratory Bulletin No. 151, 1928.

[2] In normal animals examined under high powers of the compound microscope there is a sharp contrast of gray crystals and granules against clear bluish-white cytoplasmic matrix. In brown or "bleached" animals, the brown color is not only lodged in the crystals and granules, but the cytoplasmic matrix is cloudy, more opaque and tan in color. This is doubtless due to a protein precipitation at death.

As a result of these observations, that point when the color had changed was taken arbitrarily as the death point.

COMPUTATION OF TOXICITY

In any large number of organisms subjected to the action of a toxic substance, a few individuals with low resistance will die very soon, and a corresponding number will survive an equally extended period after the death of the majority, *i.e.*, there will be a distribution of resistance similar to that found in the usual Quetelet population curve. It therefore seemed best, for purposes of comparing toxicities, to disregard the animals falling in the two extreme classes, and to consider the behavior of the middle 50 per cent only. As the total number in any one dish was 30, we therefore considered only that experimental period in which the count of living organisms was from 23 to 7. Furthermore, to express this time as a simple number for purposes of calculation, the midpoint was computed by plotting numbers against time, and observing at what hour the curve crossed a straight line drawn through the number 15. For example, in an experiment for which the results were as follows (methylamine hydrochloride M/100 and M/200):

	Amoebae alive at end of					
	2 hrs.	4 hrs.	6 hrs.	8 hrs.	10 hrs.	12 hrs.
M/100	29	21	6	1	0	
M/200	30	30	25	16	4	1

the midpoint for the M/100 series averages 4¾ hours, and for the M/200 series, 8¼ hours. A time interval in which there occurs a sharp reduction in numbers has also been designated as the period of "critical break" in the population curve. More precisely, the mortality times for the example given would be 4¾ hours and 8¼ hours respectively for the M/100 and M/200 solutions, and that interval between 4 and 6 hours in the M/100 solution would be designated as an interval of "critical break."

EXPERIMENTS AND RESULTS

Toxicity of Primary Amines

Methylamine hydrochloride.—Trial determinations made with this amine suggested the use of concentrations from M/100–M/800 at each pH. A striking contrast was observed between the gradual decrease in number of living animals in concentrations of the amine buffered to pH 6.4, and the rapid death rate in the same concentrations buffered to pH 7.8. Remarkable uniformity in the behavior of the amine was observed. With one culture,[3] for example, in concentrations of M/100 to M/800 at pH 6.4, the total count never fell to 7 over the entire 12-hour period. In a concentration of M/100 at 7.0, a critical break occurred in the count of survivors between 4 and 5 hours; in a concentration of M/200, a more gradual reduction of numbers began between 4 and 5 hours and continued for 4 hours; but in concentrations of M/400 and M/800, the total count never fell to 7. At a pH of 7.8, in concentrations of M/100 and

PROTOCOL I

Methylamine hydrochloride, M/100–M/800

	Conc. of amine	Imm. time	No. amœbæ	2 hrs.	4 hrs.	6 hrs.	8 hrs.	10 hrs.	12 hrs.
At pH 6.4	Control	8:25	30	30	29	29	29	29	29
	M/100	8:27	30	29	27	21	21	15	11
	M/200	8:30	30	29	25	25	25	21	14
	M/400	8:32	30	27	25	25	25	22	21
	M/800	8:35	30	30	30	29	29	27	26
At pH 7.0	Control	8:38	30	30	30	30	30	30	30
	M/100	8:40	30	29	21	6	1	0	
	M/200	8:42	30	30	30	25	16	4	1
	M/400	8:45	30	28	27	27	24	23	23
	M/800	8:47	30	29	29	28	26	25	22
At pH 7.8	Control	8:50	30	30	30	30	30	29	29
	M/100	8:53	30	28	8	2	2	0	
	M/200	8:55	30	19	6	1	0		
	M/400	8:58	30	22	10	6	0		
	M/800	9:00	30	23	15	8	6	3	3

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[3] For each experiment, all the animals were taken from one culture or "race." No substitutions were made from one record to another, for although the mortality-time in any one day's record shows a consistently more rapid mortality rate in the amine buffered to 7.8 than in the concentrations buffered to 6.4, the viability of the amœbæ of different cultures varies appreciably. The interchanging of records would, therefore, tend to introduce a new source of error.

M/200, the critical break in the survivor curve occurred between 2 and 3 hours; in M/400, between 3 and 4 hours, and in M/800, between 4 and 5 hours. (See Protocol I.)

Failure to reduce the number of amœbæ in the acid group to the lower limit arbitrarily set for mortality counts, *i.e.*, 7, called for repetitions of this series in higher concentrations. Experiments were therefore carried out in which M/10–M/80 concentrations of methylamine buffered to pH 6.4 were used.

Ethylamine hydrochloride.—Ethylamine hydrochloride was used in concentrations of M/100, M/200, M/400, and M/800. A typical protocol shows, as in the case of methylamine, a definite contrast between the toxicity of concentrations buffered to pH 6.4 and the same concentrations buffered to pH 7.8. The contrast between these extremes is, however, not as sharply defined as that found in the methylamine series at similar concentrations, since the arbitrary number, 7, is reached in the acid series in both concentrations of M/100 and of M/200. In the pH 6.4 series also sharp breaks occur in the two higher concentrations at a period of approximately 5 hours, though in the lower concentrations, M/400 and M/800, the number of amœbæ is not reduced to 7 during the entire 12-hour period. In the concentrations buffered to pH 7.0, sharp breaks occur at about 3 hours in the two stronger concentrations and in about 5 hours in M/400. In the weakest concentration employed the number is not reduced to 7 in the 12-hour period. At a pH of 7.8, the critical break came between 2¾ and 3¾ hours in the three strongest concentrations, and at approximately 5¾ hours in the weakest.

Propylamine hydrochloride.—Propylamine hydrochloride was used in concentrations of M/100, M/200, M/400 and M/800. A typical protocol for this amine shows that although the contrast in toxicity between concentrations buffered to pH 6.4 and to pH 7.8 is not as conspicuously evident as in the case of both methyl and ethyl amines, it is, nevertheless, readily observable. Except in the case of M/100 propylamine hydrochloride of the acid and the neutral series, the critical breaks in the count of survivals are limited to the alkaline series. In this series the mortality time for the two stronger concentrations ranges between 5 and 6¾ hours, while the death rate in the M/800 concentration is gradual, and does not reach the lower limit of the mortality count within 12 hours.

Butylamine hydrochloride.—This amine was used in concentrations of M/100, M/200, M/400 and M/800. In a typical protocol, the difference in toxicities of the acid, neutral, and alkaline series is definite. In the pH 6.4 series no critical break in resistance is shown except in the strongest concentration, M/100. The neutral series shows critical breaks in the two stronger concentrations, and a uniform gradual decrease in numbers in the other two concentrations after a period of 8–10 hours. However, in the alkaline series sharp breaks appear at about 3 to 3¾ hours in M/100, M/200; at about 6 hours in M/400 concentrations; and a rapid decrease is shown in a concentration of M/800 though the count is not reduced to 7.

Relative Toxicities of Primary, Secondary and Tertiary Amines

Primary amines.—In the previous section, no comparison was made of the relative toxicities of the four primary amines. Since each day's protocol concerned itself with the history of but 30 amœbæ, and since within so small a population the true mortality curve may not be apparent, the data from a large number of experiments were combined in order to reduce the chances of error. From typical groups of 90 to 180 amœbæ the averages given in Table I were derived.

Table I

Mortality-time at a pH of 7.8 (limit 12 hours)

	M/100	M/200	M/400	M/800
	hours	*hours*	*hours*	*hours*
Methylamine	2.91	3.00	3.16	4.16
Ethylamine	2.96	3.30	3.42	5.96
Butylamine	3.75	4.95	6.25	(Non-toxic in 12 hrs.)
Propylamine	5.10	5.86	6.75	(Non-toxic in 12 hrs.)

If the average mortality-time for any given concentration at a pH of 7.8 is taken as an indicator of toxicity, the degree of toxicity of the four primary amines may be expressed as follows: methylamine> ethylamine> butylamine> propylamine. The order of these amines on the basis of degree of toxicity does not, therefore, coincide with their order in the chemical series.

Secondary and tertiary amines.—A series of trial experiments made with dimethyl, diethyl, and trimethyl amine hydrochlorides in the concentrations used for the primary series showed at once that such concentrations were far too non-toxic to give comparable mortality-time records within a period of 12 hours. The strength of the concentrations of the secondary amines was increased to M/10, M/20, M/40 and M/80, and of the tertiary amine to M/2, M/4, M/8 and M/16. The use of such concentrations necessarily introduces new problems of degree of dissociation and osmotic pressure and the results obtained are difficult to interpret satisfactorily. In dimethylamine a certain degree of correlation with the behavior of the primary amines is observed in that the alkaline series (pH 7.8) shows the greatest degree of toxicity; critical reductions in the total count appearing in the three strongest concentrations between 2 and 4 hours, and in M/80 between 4 and 6 hours. The shrunken appearance of the amœbæ in the M/10 concentrations of the secondary amines supports the conclusion that toxicity counts of animals immersed in solutions of such high osmotic pressure are of questionable value. The abrupt fall in numbers in the trimethylamine series in concentrations of M/2 and M/4 is probably attributable to the same factor. The mortality rates in the M/8 and M/16 concentrations show a fair degree of uniformity.

Discussion

Without exception, the experimental data on the toxicities of the primary amines lend further support to the view that weak bases penetrate more rapidly from alkaline than from acid solutions. The possibility must be considered that the increased toxicity is an expression of some direct action by the more basic media on the cells themselves. This seems unlikely since it was noted that the viability of the amœbæ was approximately equal in the control solutions at all of the H-ion concentrations used. There remains, then, the consideration of whether the changes in degree of dissociation of the amines are quantitatively related to the degree of toxicity produced.

In a M/30 solution of methylamine hydrochloride at a pH of 6.4, the "lethal time" for one culture of amœbæ was 4.25 hours. The amount of free base present (using the value 5×10^{-4} for the dissociation constant) is found to be 1.65×10^{-6}. Equivalent amounts of free base are present in a M/121 solution at a pH of 7.0, and in a M/763 solution at a pH of 7.8. Experimental data give (for the same culture) the same lethal time at a pH of 7.0 in a M/100 concentration, and, at a pH of 7.8, in a M/800 concentration. The parallel between the increase in amount of undissociated base and the decrease in length of the toxicity time is striking.

In two series of ethylamine hydrochloride immersions the lethal time, at a pH of 6.4, in a M/100 solution was 5.75 hours, and in a third series, 6.5 hours. The concentration of free base present (using the value 5.6×10^{-4} for the dissociation constant of the amine) is 0.44×10^{-6}. At a pH of 7.0, an equivalent amount is present in a M/407 solution, and at a pH of 7.8, in a M/2539 solution. Experimental data show, even in the presence of a great viability difference in the three races (cultures) used, very consistent results. At a pH of 7.0, in the first two series, the lethal time 5.75 hours was reached in the M/400 solutions, and in the third series the lethal time 6.5 hours is found in this same concentration. However, at a pH of 7.8, though the concentration toxic in 6 hours is M/800 for all three series, this is in wide disagreement with the expected figure, *i.e.,* M/2539.

In the propylamine hydrochloride series the divergence between calculated and actual concentrations is even more pronounced. Agreement in behavior of resistant and weak races (cultures) is, however, again seen to be strikingly consistent. In two races with lethal times of 7 hours and 6.5 hours respectively in M/100 solutions at a pH of 6.4, the same lethal periods were found at pH 7.0 in M/150 solutions, and at pH 7.8, in M/400 solutions. Calculations made on the basis of 4.7×10^{-4} as the dissociation constant, gave the expected figures as follows: for a pH of 7.0, M/401; for a pH of 7.8, M/2539. This discrepancy in the case of propylamine clearly indicates the activity of some important and unknown factor.

No values for the dissociation constant of butylamine can be found in the literature.

Under the conditions of these experiments it appears that although a close relationship exists between the amount of undissociated base present and the degree of toxicity of methyl and ethylamines, this agreement does not hold true for propylamine.

Conclusions

1. Under the conditions of the experiment, the degree of toxicity of solutions of the hydrochlorides of methyl, ethyl, propyl and butyl amines varies uniformly with the H-ion concentration as follows: the toxicity at a pH of 7.8> at a pH of 7.0> at a pH of 6.4.

2. In solutions of methylamine hydrochloride, the parallel between the increase in amount of undissociated base and the decrease in length of toxicity time is striking. In ethylamine solutions, this relation holds in concentrations buffered to a pH of 6.4 and 7.0, but not for the solutions buffered to a pH of 7.8. In the case of propylamine, though all the experimental data are convincingly uniform, the divergence between calculated and actual toxicity concentrations is even more pronounced. The presence, therefore, of some other important factor or factors is clearly indicated.

3. The relative degree of toxicity of the first four primary amines may be expressed as follows: methylamine> ethylamine> butylamine> propylamine.

4. Solutions of the hydrochlorides of dimethyl and diethyl amines were found to be less toxic than methyl and ethyl amines.

5. In the case of diethylamine, there is a certain degree of correspondence with the behavior of primary ethylamine, in that the alkaline series (at a pH of 7.8) shows the greatest degree of toxicity.

6. Solutions of triethylamine are still less toxic than those of the secondary ethylamine. To obtain mortality-time records within twelve hours, the effective concentration of tertiary ethylamine reaches a point where the high osmotic pressure causes shrinkage of the cells.

We wish to express our thanks to Dr. Kenneth C. Blanchard, of Washington Square College, for his valuable criticism, and to Dr. Samuel E. Hill, of Rockefeller Institute, for his interest in checking our calculations.

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STUDIES ON THE CILIATES FROM FRESH WATER MUSSELS

II. The Nuclei of Conchophthirius Anodontæ Stein, C. Curtus Engl., and C. Magna Kidder, during Binary Fission

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In a previous paper (Kidder, 1934) I have given a description of the general morphology, neuromotor apparatus, and vegetative nuclei of the three species of *Conchophthirius* to be discussed in the following pages. Mention was made of the occurrence of binary fission, and the general appearance of the metaphase spindles was figured. It is the purpose of the present paper to report the cytological details of the nuclear phenomena during division and to point out differences from and similarities to the nuclei of related forms.

Relatively few of the many descriptions of ciliate division give a detailed account of the number and behavior, during binary fission, of the micronuclear chromosomes. Especially noticeable is the general lack of close observations as to the period between the metaphase and the anaphase stages with regard to the method of chromosomal division. This situation is quite obviously due to the difficulty of observation of so small a cell element and also in part to the usually short duration of this stage. Whether ciliate chromosomes divide transversely or longitudinally is of great theoretical significance, as was clearly pointed out by Calkins (1930*a*). A great many more observations on this point are needed before we can formulate any general conclusions.

Due to the large size of the micronucleus of *Conchophthirius anodontæ*, the great abundance of material, and the ease of fixing and staining, I have been able to make a detailed study of the chromosomal division in this species. Although the other two species studied, *C. curtus* and *C. magna,* were very plentiful, their micronuclei are so small, as pointed out before (Kidder, 1934), that I was unable to observe the chromosomes clearly and so could not be sure of the exact method of chromosomal division. In all three species the changes occurring in the macronuclei during fission could be followed with ease.

The material used in this study was obtained from the same sources as those previously reported (Kidder, 1934). The preparations were made at the Marine Biological Laboratory at Woods Hole, Massachusetts.

TECHNIQUE

The method of obtaining the ciliates for study of the division process was similar to that used in the study of *Conchophthirius mytili* (Kidder, 1933). As in that species, the present ciliates can be selected for fixation by observing, through a dissecting binocular, the condition of the macronucleus. In this manner practically any stage can be obtained in a very short time. However, unlike *Conchophthirius mytili*, the present ciliates are so numerous that a single mussel will often yield all of the various stages of binary fission.

The fixatives employed were Flemming's, Schaudinn's, Bouin's, and Gilson-Carnoy's fluids and sublimate-acetic in 95 per cent alcohol. Flemming's fluid was followed by bleaching in H_2O_2.

The stains giving uniformly good results were Heidenhain's and Delafield's hæmatoxylins, the Borrel stain and the Feulgen thymonucleic acid reaction. By far the best preparations were obtained by the Feulgen reaction following Schaudinn's fluid or sublimate-acetic in 95 per cent alcohol, and Heidenhain's hæmatoxylin (long method) after Schaudinn's or Flemming's fluids.

I employed a modification of the picric acid destaining method of Tuan (1930) following Heidenhain's hæmatoxylin. For this modification I am indebted to Messrs. T. T. Chen and R. Wichterman. After staining, the coverglasses are rinsed in water and placed in a saturated aqueous solution of picric acid. Usually twenty to thirty minutes are required to extract a sufficient amount of stain. Critical examination can be made at any time during the destaining. By passing the coverslip over the mouth of a bottle containing concentrated ammonium hydroxide the brownish appearance imparted by the picric acid is removed. The fumes change the brown of the picric to a bluish black and render the organism quite clear. Care should be taken not to expose the coverslips to the ammonium hydroxide for a longer period than is needed to produce the bluish color, as excess will result in the swelling of the organism. When sufficient stain has been extracted, so that the cytoplasm is a pale gray, the coverglasses are washed in running water for thirty minutes, dehydrated and mounted.

OBSERVATIONS

Conchophthirius anodontæ Stein

Micronucleus.—The first sign of division in this ciliate is to be found in the swelling and loss of staining capacity of the micronucleus.

From the compact vegetative condition the chromatin becomes flocculent and the nuclear membrane moves away from the central mass leaving a clear area. This clear area is no doubt partly the result of shrinkage brought about by fixation. The micronucleus moves out of its pocket in the macronucleus and takes up a position in the mid-region of the cell. The chromatin becomes irregularly disposed in a reticulate fashion (Fig. 1, *A*). Swelling continues and the chromatin condenses into a twisted band, which at first is quite irregular. This is similar to a spireme stage. (Fig. 1, *B*). I am unable to say whether the spireme band is continuous or broken at this time. In the clear space at the poles of the chromatin mass delicate spindle fibers can be seen. These fibers appear to be pushing out from the central matrix. The spireme band becomes more basophilic and breaks into many small segments. These segments orient themselves along the long axis of the nucleus, with their ends pointing toward the rapidly forming spindle fibers (Fig. 1, *C*). The whole nucleus is undergoing elongation. The chromatin making up the bands is disposed in irregularly spherical chromomeres of considerable size. In carefully differentiated material it is possible to count these bands even at this early stage. The number is twelve. The metaphase plate is formed by a condensation of the twelve bands into definite but rough chromosomes, a concomitant elongation of the nucleus, and completion of the division spindle (Fig. 1, *D*). The chromosomes of the metaphase plate are always lined up along the long axis of the spindle.

I have never seen any evidence of an endosome at any stage in the micronuclear mitosis, such as was found by Wenrich (1926), Manwell (1928) and Turner (1930).

About this time the first indication of actual chromosomal division may be seen. A longitudinal split occurs in each chromosome and eventually the two daughter chromosomes are clearly discernible (Fig. 1, *E*). Material stained after the Feulgen reaction was the most satisfactory for this stage as the chromosomes are sharply outlined and there is none of the haziness so often seen in material stained in hæmatoxylin. Unlike the majority of ciliates, the metaphase appears to be of rather long duration and dozens of well-prepared individuals were obtained for study. In no case, however, was I able to detect the split in all the chromosomes at the same time. The metaphase plate is quite regular and all the chromosomes are of approximately equal length. The migration of the daughter chromosomes to their respective poles is again a relatively slow process, judging from the great number of anaphases obtained. The daughter halves slip by one another resulting in a broad band of chromosomes in the central portion of the spindle. Each daughter increases somewhat in size. It is possible at all times during

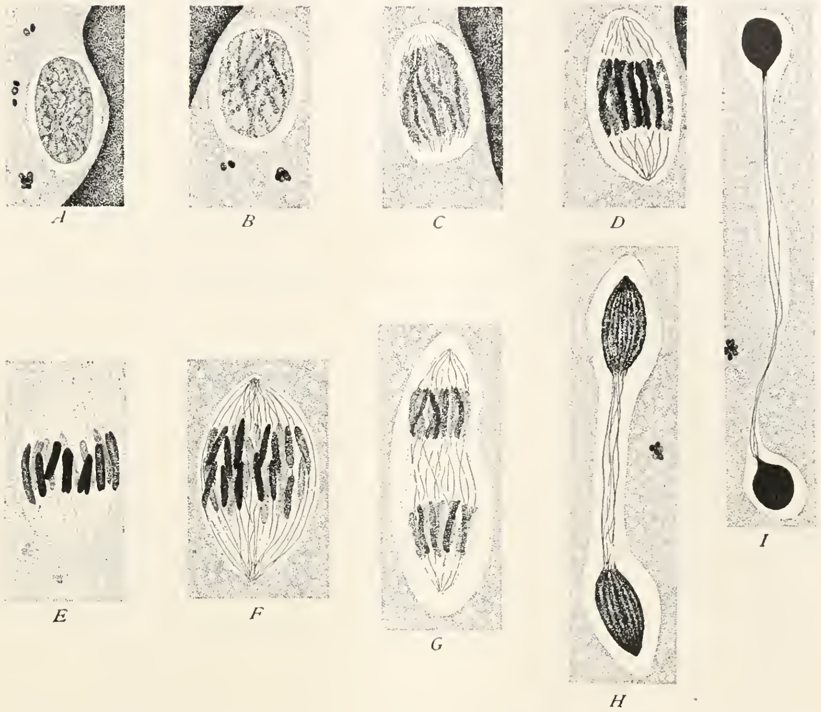

FIG. 1. The micronucleus of *Conchophthirius anodontæ* during mitosis. All except *E* were fixed in Schaudinn's fluid, stained with Heidenhain's hæmatoxylin and destained in aqueous picric acid. *E* was fixed in sublimate acetic in 95 per cent alcohol and stained after the Feulgen reaction. Drawings were made with the aid of a Promar projector. × 2644.

A. Early prophase with the chromatin in the flocculent state.

B. Spireme stage. The spindle fibers are just beginning to appear.

C. Late prophase. Twelve long strands are oriented toward the poles of the forming spindle.

D. Early metaphase. Twelve chromosomes.

E. Metaphase. Twelve fully contracted chromosomes. Some show definite longitudinal splits while in others only the notched condition of the ends indicates where the split will appear.

F. Early anaphase. Daughter chromosomes migrating towards their respective poles.

G. Late anaphase. The two groups of twelve chromosomes each are well separated.

H. Early telophase.

I. Late telophase. Separation spindle clearly shown.

this slipping-past process to count the twenty-four daughter halves (Fig. 1, *F*). Not only is it possible to make consistent counts but because of their proximity it is usually possible to detect which two halves were originally a single chromosome. The metaphase split and the early anaphase migration are undoubtedly the most interesting features of this extremely large and clear micronucleus. I have never seen any indication of the dumb-bell-shaped chromosomes of the early anaphase that occur so frequently in ciliates, and because of the extreme numbers of all stages of mitosis available for study, the large size, and the clear staining properties of this micronucleus, I feel certain that the activity described above is accurate as far as *Conchophthirius anodontæ* is concerned.

The late anaphase is quite regular. Twelve daughter chromosomes migrate to the poles of the now elongated spindle (Fig. 1, *G*). Here they contract into a typical spear-head mass. The fibers of the spindle retain their form between the two daughter nuclei (Fig. 1, *H*) even to the very late telophase. The formation of the daughter nuclei is regular, the chromatin contracting into two homogeneous spheres (Fig. 1, *I*). As the daughter nuclei move apart a long " separation spindle " is pulled out between them. In the flared portion of the separation spindle a number of fibers persist for some time. The separation spindle and contained fibers gradually fade out and the two micronuclei round up and take up their positions against the daughter macronuclei.

Macronucleus.—During the early prophases of the micronucleus, the macronucleus of *Conchophthirius anodontæ* swells slightly and, as the late prophase of the micronucleus sets in, migrates to a central position in the cell. During the metaphase of the micronucleus, the macronucleus elongates in the direction of the long axis of the cell and internal changes take place. The chromatin becomes evenly granular and as constriction starts an area of more deeply staining chromatin forms in the central region. This island is marked off from the major portion of the macronucleus by a halo of less deeply staining chromatin (Fig. 2, *A*). The differentiation of the central area is not the same in all individuals, for in some the nuclear granules appear evenly distributed until a later stage. The central mass is the residual chromatin characteristic of the Conchophthiriidæ. The residual mass condenses into a densely staining sphere between the dividing daughter halves of the macronucleus (Fig. 2, *B*). The macronuclear membrane flares out to accommodate the residual mass just as it does in *Conchophthirius mytili* (Kidder, 1933*a*) and *Ancistruma isseli* (Kidder, 1933*b*). Subsequent pulling apart of the daughter halves leaves the residual mass near the cell center (Fig. 2, *C*). Finally the connections break and the residual mass rounds up

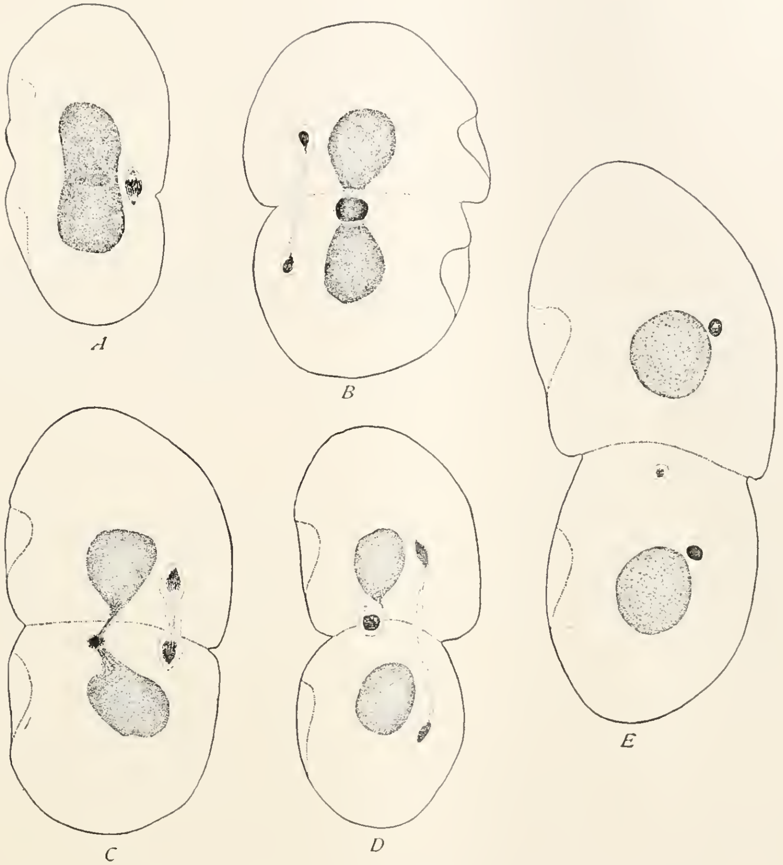

Fig. 2. The macronucleus of *Conchophthirius anodontæ* during binary fission. Camera lucida drawings. × 511.

A. Earliest evidence of the formation of the ball of residual chromatin. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.

B. Residual ball somewhat contracted and lying between the separating halves of the macronucleus. Schaudinn's fluid—Heidenhain's hæmatoxylin.

C. Further contraction of the residual ball and separation of the macronuclear halves. Schaudinn's fluid—Heidenhain's hæmatoxylin.

D. Daughter macronuclei have separated. Residual ball has rounded up within a fragment of the old macronuclear membrane. Gilson-Carnoy's fluid—Delafield's hæmatoxylin.

E. Plasmotomy well under way. Residual chromatin is fading out in the cytoplasm. Sublimate acetic—Feulgen reaction.

(Fig. 2, *D*). Plasmotomy proceeds and the residual chromatin, in the cytoplasm of either of the daughter ciliates, starts to disintegrate and rapidly loses its affinity for basic stains (Fig. 2, *E*) until finally it becomes completely absorbed. Plasmotomy may or may not be completed before the entire dissolution of the residual chromatin has occurred.

In a few cases of division in this species no residual chromatin was visible, the macronucleus dividing cleanly. These cases were similar to the reorganized exconjugants of *Conchopththirius mytili* (Kidder, 1933*a*). I believe it highly possible that the cases of cleanly dividing macronuclei of *Conchophthirius anodontæ* also represent organisms shortly after conjugation. My belief is based on the presence of considerable numbers of reorganizing individuals found during the first month of this investigation (June, 1933), indicating that conjugation had previously taken place. I will have more to say on this matter in a later paper.

Irregularities of division, which I am inclined to regard as abnormal and probably pathological, are occasionally encountered. A few dividing organisms showed an obvious upset in the normal procedure. In some cases the macronuclear division was very asymmetrical, the greater portion being included in one daughter organism (Fig. 7, *A*). In others the nuclear division failed to keep pace with plasmotomy, all of the chromatin remaining in one daughter, leaving the other devoid of nuclei (Fig. 7, *B*). In still other cases only a small portion of the macronucleus is passed to one daughter ciliate while the other daughter retains most of the macronuclear material and all of the micronuclear material (Fig. 7, *C*). In some of these cases we have visible evidence of the possibility of the formation, through faulty fission, of an amicronucleate race. Whether or not this ever occurs, or indeed whether or not either daughter is viable, I cannot say.

Conchophthirius curtus Engl.

Micronucleus.—The general activity of the micronucleus of *Conchophthirius curtus* is very similar to that of *C. anodontæ* but because of its small size it is impossible to follow the finer details of exact chromosome number and method of chromosomal division, at least in my material.

The early prophase occurs as the micronucleus emerges from its pocket in the plastic macronucleus. It swells slightly, the chromatin becoming loosened and finely granular and less deeply staining than in the vegetative state (Fig. 3, *A*). The micronucleus now elongates, always in the plane of the long axis of the body of the ciliate. The chromatin becomes condensed into many granular threads, lined up along the long axis (Fig. 3, *B*). These threads contract, increase in staining capacity, and form the chromosomes of the metaphase plate (Fig. 3, *C*). In the meantime spindle fibers have pushed out to form a rather sharply pointed spindle. The chromosomes lie very close to one another and I have not been able to make accurate counts, even after the Feulgen reaction. The next stage is not clear. It is very hard to determine whether a broad band of chromosomes represents a contraction from the prophase or a migration at early anaphase. Of this I am certain, however, that in the dozens of cases of this stage studied I have never found the dumb-bell-shaped chromosomes such as were found in *Conchophthirius mytili* (see Plate II, Fig. 16 in Kidder, 1933*a*). This would lend support to the view that chromosomal division is longitudinal as in *C. anodontæ,* and that in the early anaphase migration the two daughters slip past one another.

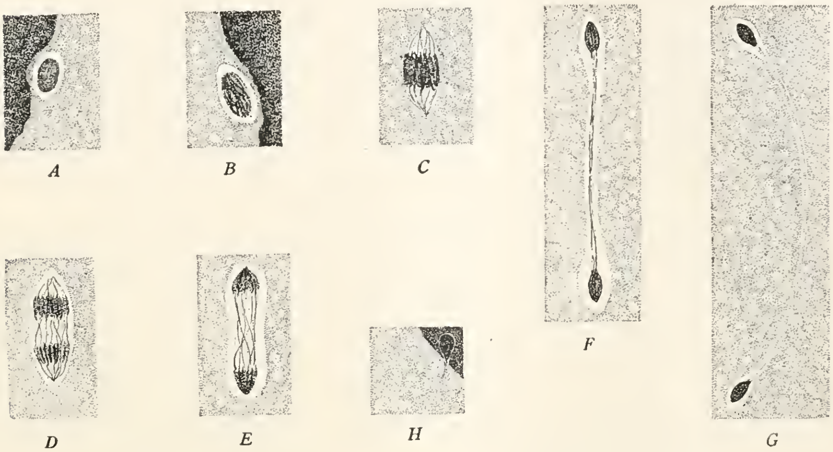

FIG. 3. The micronucleus of *Conchophthirius curtus* during mitosis. Camera lucida drawings. × 2663.

A. Early prophase. Micronucleus migrating from its pocket in the macronucleus. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.

B. Prophase. Chromatin in strands. Sublimate acetic—Feulgen reaction.

C. Metaphase. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.

D. Anaphase. Schaudinn's fluid—Heidenhain's hæmatoxylin.

E. Early telophase. Schaudinn's fluid—Heidenhain's hæmatoxylin.

F. Telophase. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.

G. Later telophase. Schaudinn's fluid—Heidenhain's hæmatoxylin.

H. Reconstruction of one daughter micronucleus. Evidence of separation spindle still seen. Sublimate acetic—Heidenhain's hæmatoxylin.

The later anaphase is quite clear. Two groups of evenly distributed chromosomes are seen toward the poles of the spindle (Fig. 3, *D*). Between the two daughter groups of chromosomes the mid-fibers are always quite clear. Further elongation of the micronucleus and further migration of the daughter chromosomes completes the anaphase (Fig. 3, *E*). Very shortly the chromosomes lose their identity in the spearhead telophase chromatin mass (Fig. 3, *F*). These daughter micronuclei pull further and further apart, stretching out an extremely long connecting strand, composed of the mid-fibers and the nuclear membrane (Fig. 3, *G*). It is usually not until the daughter micronuclei have entered the daughter macronuclei that this connection is ruptured. For some time thereafter traces of the connecting strand can be seen extending from the otherwise contracted micronuclei (Fig. 3, *H*).

Macronucleus.—The phenomena accompanying the division of the macronucleus of *Conchophthirius curtus* so closely resembles that of *C. anodontæ* that I shall not dwell at length on this subject.

The furrowed macronucleus migrates into the mid-region of the cell and elongates. A heavily staining ball of chromatin becomes differentiated from the rest of the macronuclear chromatin in the central region. This ball is surrounded by a less densely staining halo (Fig. 4, *A*). As the macronucleus constricts the ball contracts (Fig. 4, *B*) and remains between the daughter macronuclei (Fig. 4, *C*). The residual mass retains its connections with the daughter macronuclei for some time (Fig. 4, *D*). It is finally freed and then rounds up in the cytoplasm of either daughter ciliate (Fig. 4, *E*). The daughter macronuclei lose their smoothly granular condition and again become furrowed and the residual chromatin disintegrates and disappears.

I have found several cases where no residual chromatin was formed, the macronuclei separating cleanly. As in *C. anodontæ* and *C. mytili* I am inclined to regard these cases as ciliates shortly after exconjugant reorganization. I have found numerous conjugating pairs and many reorganizing individuals, and the cases in which a residual mass was lacking were in about the proportions one might expect if these cases do represent exconjugants. Future investigation on the conjugation of these ciliates may shed more light on this interesting problem.

Abnormalities of fission were rarely encountered in this species. Three cases of precocious plasmotomy were observed where the macronucleus and the micronucleus were included in a single daughter, the other daughter being entirely devoid of nuclear material (Fig. 7, *D*).

Conchophthirius magna Kidder

Micronucleus.—As I have previously stated (Kidder, 1934), *Conchophthirius magna* possesses, in about 90 per cent of the cases, two small, compact vegetative micronuclei. Dividing individuals, in which the micronuclei are quite easily seen, confirmed this percentage. About 10 per cent of the individuals possess but one micronucleus. In two cases,

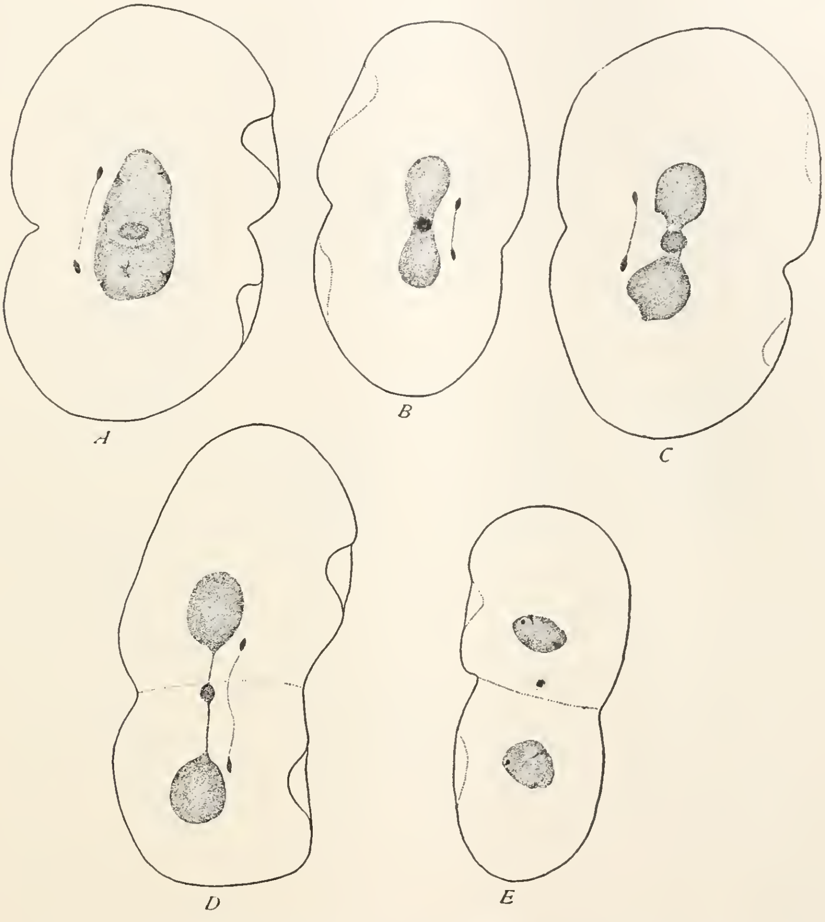

FIG. 4. The macronucleus of *Conchophthirius curtus* during binary fission. Camera lucida drawings. × 521.

A. Formation of the ball of residual chromatin. The macronucleus is still in a furrowed condition. Schaudinn's fluid—Heidenhain's hæmatoxylin.

B. Constriction of macronucleus. Residual chromatin quite compact. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.

C. Further constriction of the macronucleus and separation of the residual chromatin. Flemming's fluid—Heidenhain's hæmatoxylin.

D. Residual chromatin connected to daughter macronuclei. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.

E. Daughter macronuclei reorganized. Residual chromatin disintegrating. Sublimate acetic—Feulgen reaction.

however, I was unable to see any micronuclei. These individuals, though in the early stages of fission, contained an enormous amount of food which, I believe, obscured the spindle or spindles that may have been present.

The micronuclei of *C. magna* appear to possess a greater tendency to remain near or in contact with the macronucleus during the prophase, metaphase and anaphase stages than do those of *C. anodontæ* and *C. curtus*. Swelling of the micronuclei results in a finely granular condition at the onset of mitosis (Fig. 5, *A*). The chromatin becomes oriented into granular threads extending nearly the entire length of the somewhat elongated nucleus (Fig. 5, *B*). These threads contract and form rather definite chromosomes on the equatorial plate of the now fully formed spindle (Fig. 5, *C*). These chromosomes are again too small and too compact to enable one to count them. The beginning of the anaphase is again, as in *C. curtus*, a question of interpretation. I believe Fig. 5, *D*

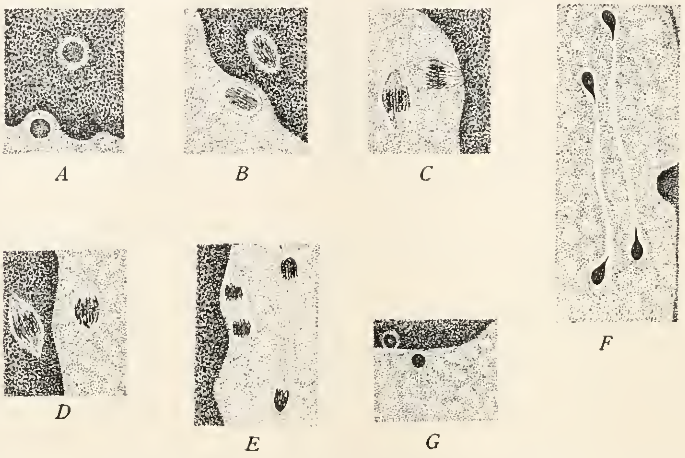

FIG. 5. The micronuclei of *Conchophthirius magna* during mitosis. Camera lucida drawings. × 2076.

A. Early prophase. Gilson-Carnoy's fluid—Feulgen reaction.

B. Late prophase. Sublimate acetic—Heidenhain's hæmatoxylin.

C. Metaphase. Sublimate acetic—Feulgen reaction.

D. Early anaphase. Schaudinn's fluid—Feulgen reaction.

E. One micronucleus in middle anaphase and one in late anaphase. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.

F. Telophase. Gilson-Carnoy's fluid—Feulgen reaction (counterstained in Borrel II).

G. Late telophase. The micronuclei are compact and spherical but still connected to the long separation spindles. Schaudinn's fluid—Heidenhain's hæmatoxylin.

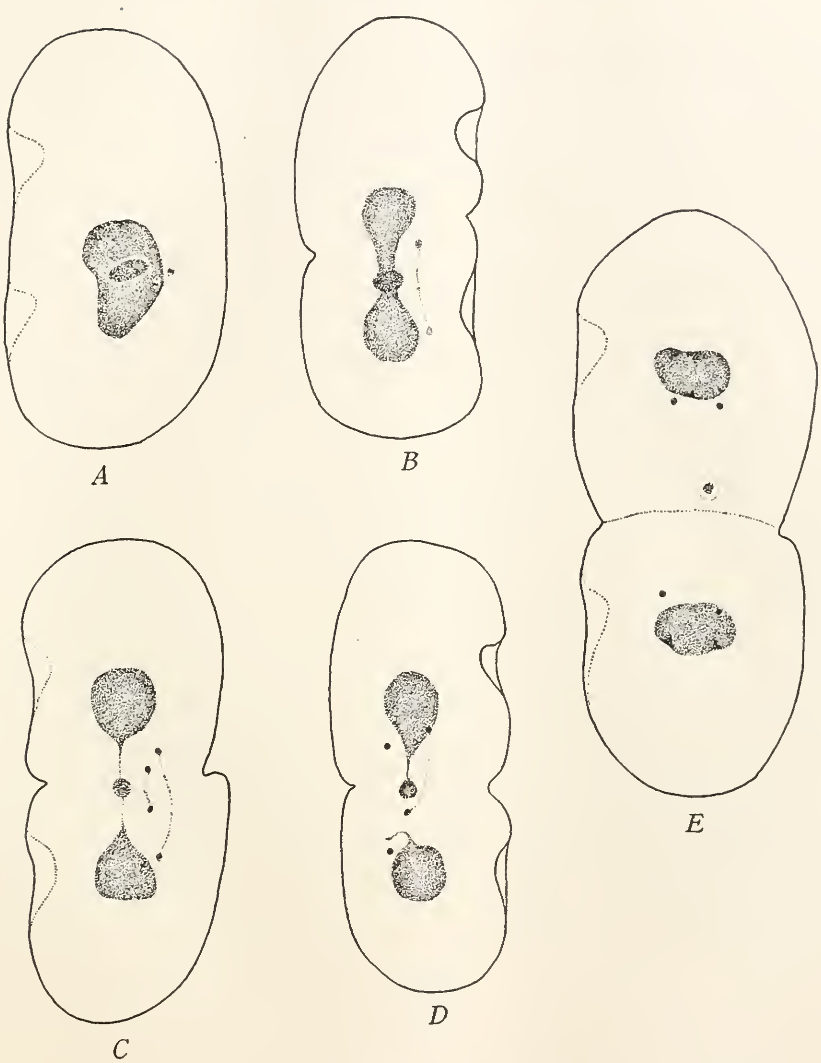

Fig. 6. The macronucleus of *Conchophthirius magna* during binary fission. Individuals were all fixed in sublimate acetic in 95 per cent alcohol and, with the exception of *E*, were stained after the Feulgen reaction. *E* was stained in Heidenhain's hæmatoxylin. Camera lucida drawings. × 350.

A. Formation of ball of residual chromatin.

B. Constriction of macronucleus and separation of residual ball. The individual possessed a single micronucleus.

C. Separation of daughter macronuclei.

D. Separation of one daughter macronucleus from residual ball.

E. Reorganization of daughter macronuclei and disintegration of residual chromatin.

represents the slipping past of daughter chromosomes on their way to their respective poles. I have never seen any dumb-bell-shaped chromosomes that would indicate transverse division.

The later anaphases are quite regular and very similar to those of *C. curtus* (Fig. 5, *E*). The mid-fibers are clearly demonstrated between the two daughter groups of chromosomes. In the telophase the daughter

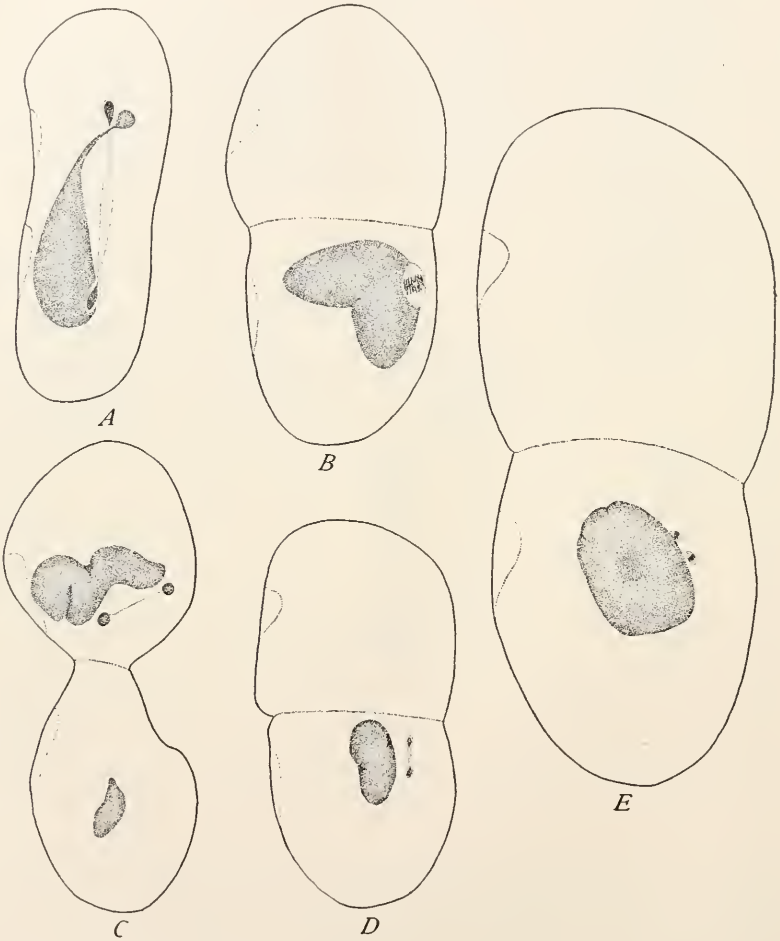

FIG. 7. Abnormal divisions. × 486.

A. *Conchophthirius anodontæ*. Schaudinn's fluid—Feulgen reaction.
B. *C. anodontæ*. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.
C. *C. anodontæ*. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.
D. *C. curtus*. Sublimate acetic—Heidenhain's hæmatoxylin.
E. *C. magna*. Bouin's fluid—Heidenhain's hæmatoxylin.

micronuclei pull out a long connecting strand (Fig. 5, *F*) which, in some cases, persists until the four compact daughter micronuclei enter the macronuclei (Fig. 5, *G*).

Macronucleus.—As the micronuclei prepare for mitosis the macronucleus of *Conchophthirius magna* begins to lose its furrowed condition and finally becomes evenly granular. A large mass of deeply staining chromatin, surrounded by a lightly staining halo, is differentiated near its center (Fig. 6, *A*). This mass contracts into a ball and is left in the division plane as the daughter halves of the macronucleus pull apart (Fig. 6, *B*). Long connecting strands persist for some time (Fig. 6, *C*) as in the preceding species. When these strands are severed it is a common occurrence to find one or both curved back on itself (Fig. 6, *D*) as if they had been suddenly released from a stretching force. As the daughter macronuclei become reorganized in the daughter ciliates the residual chromatin rounds up and degenerates (Fig. 6, *E*), being absorbed into the cytoplasm of that daughter cell in which it lies.

These phenomena exactly parallel those of *Conchophthirius anodontæ* and *C. curtus*.

So far I have never encountered dividing specimens of *C. magna* lacking the residual chromatin, nor have I ever seen any indications of conjugation, either conjugating pairs or reorganizing individuals. This is evidence, I believe, for my above view, that the lack of the residual chromatin in these forms is in some way connected with the reorganization of the macronuclear chromatin following conjugation, a view more firmly substantiated in the case of *C. mytili* (Kidder, 1933*a*).

A very few abnormal divisions were encountered where the nuclear apparatus lagged in its division behind the rest of the cell (Fig. 7, *E*). In these cases the spindles and macronuclei appeared normal, except for their position in relation to the division plane, while cytoplasmic reorganization was far in advance.

Discussion

In reviewing the literature on micronuclear mitosis in ciliates one is at a loss to decide just what type of chromosomal division takes place in the majority of forms. It would seem that either transverse or longitudinal occurs even in closely related species. Of course if one is to accept the contention of Calkins (1930*a*) that within the protozoan nucleus there is only one type of gene per chromosome, then this situation would lose its significance. As Calkins points out, it would then be immaterial whether the chromosomes divided longitudinally or transversely.

To review briefly a few of the cases on record one must note that Stevens (1903) says that the four vegetative chromosomes in the micronucleus of *Boveria subcylindrica* divide transversely. This statement is repeated in a later paper (Stevens, 1910). Her figures indicate a pinching in two in the central portion of each long chromosome.

Calkins (1919) says that the vegetative chromosomes of *Uroleptus mobilis* line up with their long axes parallel to the long axis of the spindle. His figures clearly show this condition. But as to the actual division he says " Whether these rods are divided transversely or longitudinally cannot be determined owing to their minute size and densely packed condition " (p. 306).

In the case of *Uroleptus halseyi* the micronuclei are extremely large and, according to Calkins (1930*a*), the vegetative chromosomes divide in a transverse manner. His figures do not show the actual fission of the chromosomes, however, but merely the metaphase plate and later the two anaphase groups.

In *Conchophthirius mytili* (Kidder, 1933*a*) the sixteen well-formed vegetative chromosomes pull out into dumb-bell-shaped bodies at early anaphase and it certainly appears that division could only have been accomplished in a transverse manner.

Turner (1930) figures the eight vegetative chromosomes of *Euplotes patella* in the early anaphase as dumb-bell-shaped bodies. He says " Whether the chromosomes divide longitudinally or transversely has not been definitely established. Their appearance in the early anaphase would indicate that the latter case were true. As the two halves of a chromosome separate, there seems to be a fine connection between them for a short time which soon breaks. This observation is evidence against, but does not exclude the possibility that the chromosomes split longitudinally. The fact that these chromosomes are never seen in any other position than parallel to the long axis of the spindle indicates that if they do split longitudinally the daughter halves must slip past each other rather than that one or both revolves on the end of the other " (p. 210). This last observation is very interesting in the light of conditions here described for *Conchophthirius anodontæ* where an actual slipping past is demonstrated. Turner's prediction was, no doubt, influenced by his finding of the daughter chromosomes slipping past one another in the third maturation division of *E. patella*.

Manwell (1928) states that the vegetative chromosomes of *Pleurotricha lanceolata* appear to split longitudinally and, in the early anaphase, draw out into V-shaped bodies with connecting bands. His figures indicate only the V-shaped chromosomes in the process of migration. No figure of the actual split is given.

One clear case of longitudinal chromosomal division is given by Chen (1932) in a preliminary report on the mitosis in *Zelleriella.* Here the chromosomes form a rather irregular metaphase plate and plainly divide throughout their length. He states ". . . the longitudinal split of the chromosomes shows clearly and the chromatids or daughter halves of each chromosome can be identified" (p. 270). This species, however, is a member of the Opalinidæ and does not possess the typical dimorphic nuclei of the majority of ciliates and it is, therefore, a comparison of questionable value.

From the above citations it is evident that as far as we know at present the micronuclear chromosomes of ciliates may divide either transversely or longitudinally. In the former method the chromosomes pull into dumb-bell-shaped bodies; in the latter no such dumb-bells are formed.

The chromosomes of *Conchophthirius anodontæ,* and perhaps those of *C. curtus* and *C. magna,* fall into the latter category. The longitudinal split and the slipping past of the daughter halves in the early anaphase have been encountered too frequently in my material to allow for an alternative interpretation. Only future investigations of ciliates possessing large micronuclei with relatively few chromosomes will determine how widespread is this method of chromosomal division.

It is becoming more and more apparent that the macronuclear chromatin is intimately bound up in the reorganization process of the ciliate cell, not only after conjugation and endomixis but during and after binary fission. As regards the extrusion of macronuclear chromatin into the cytoplasm my observations on the three species of *Conchophthirius* described above are parallel to those of Behrend (1916) on *Loxocephalus;* MacLennan and Connell (1931) on *Eupoterion pernix* (figured but not described); Kidder (1933*a*) (1933*b*) on *Conchophthirius mytili* and *Ancistruma isseli;* and recently Haas (1933) on the divisions within the cyst of *Ichthyophthirius multifiliis.* Rossolimo and Jakimowitsch (1929) describe the casting out of granular masses from the seven macronuclei of *Conchophthirius steenstrupii* during binary fission. It seems probable that these masses represent extrusion chromatin although the authors place a rather different interpretation upon them. Their conclusions are based solely upon the staining reactions of these masses after the use of iron hæmatoxylin.

I am inclined to believe that the extrusion chromatin, regularly given off from the macronuclei of the above forms, must represent waste substances of prolonged cell metabolism. Calkins (1930*b*) called the regular extrusion of chromatin from the macronuclei of *Uroleptus halseyi* prior to fission a "purification process." So regular and widespread a phenomenon certainly cannot be without meaning, and it seems possible that the reorganization process that takes place in the macronuclei of the hypotrichous ciliates is merely a different method of accomplishing the same end—the elimination of worn-out substances.

Summary

1. The nuclear phenomena incident to fission are described for three ciliate commensals of fresh water mussels, *Conchophthirius anodontæ, C. curtus,* and *C. magna.*

2. The micronuclear chromatin of *C. anodontæ* forms a granular spireme in the prophase. From this spireme form twelve distinct rod-like chromosomes. On the metaphase plate each chromosome splits longitudinally. In the early anaphase the daughter halves slip past one another and form two groups of twelve chromosomes each. The late anaphase and the telophase are quite regular, the compact daughter micronuclei forming from the twelve daughter chromosomes.

3. The macronucleus of *C. anodontæ* undergoes fission, throwing out a deeply staining ball of chromatin near the division plane. This residual chromatin disintegrates and is absorbed into the cytoplasm.

4. *C. curtus* and *C. magna* parallel *C. anodontæ* in all nuclear activity during fission. The micronuclei are, however, too small to permit the observation of minute details.

5. Several cases of abnormal divisions are reported, occurring in all three species. In these cases it would appear that the synchronization of cytoplasmic and nuclear activity had, in some manner, become disorganized.

6. A short review of the literature dealing with ciliate fission, particularly that concerning micronuclear mitosis and the extrusion of macronuclear chromatin, is given.

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ANALYSIS OF THE REGENERATIVE PROCESSES IN NEMERTEANS

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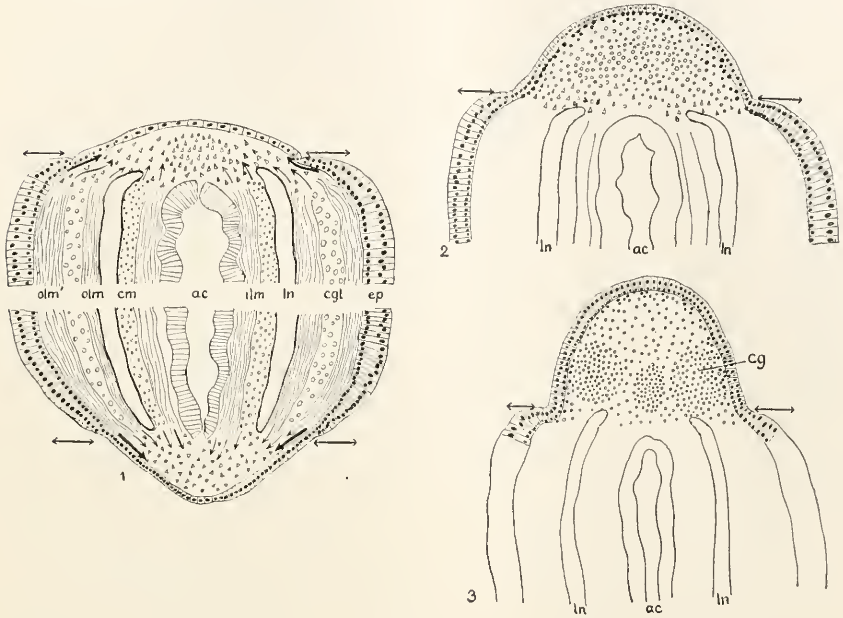
Because of the sharp demarcation of their tissues, the nemerteans are particularly favorable for a study of the cellular changes which occur during the restoration of the new individual from a fragment of the body. In some species, such as *Lineus socialis,* the regenerative potency is so great that almost any small piece of the body, provided it contains a portion of one of the lateral nerve cords, is able to develop into a minute worm of normal proportions. The persistence of this regenerative ability is demonstrated by repeatedly cutting off portions of partially regenerated pieces until extremely minute individuals less than a hundred thousandth the size of the original are finally obtained (Coe, 1929). The cellular activities involved in such regeneration have already been described (Coe, 1934), and we may now inquire as to the organizing agencies which are responsible for them.

In the regeneration of a fragment of the body of *Lineus socialis* it is evident that the stimulus of the changed internal environment causes a vigorous contraction of the body and a coördinated migration of epidermal and connective tissue cells toward both the cut surfaces. The wounds are thereby closed and healed (Fig. 1).

The cuts also activate the dormant cells which are situated in the parenchyma between the organ systems in all parts of the body. By virtue of their activation these cells assume the properties of regenerative cells. They migrate both anteriorly and posteriorly, finally collecting in large numbers beneath the new epidermis which has already covered the cut ends of the fragment.

Those that migrate posteriorly are soon incorporated into the organ systems of the original body. These organs were injured by the cut and their tissues are in process of restoration, both by the multiplication of the differentiated cells and the incorporation of new ones. The fate of each of the migratory cells is presumably determined by the differentiated cells which it reaches. The functional tissues thus organize the new posterior extremity.

Posterior regeneration is therefore closely similar to normal growth, although phagocytosis is more extensive in removing unassimilable elements and more essential in the nutrition of the regenerating tissues than is the case in normal growth.



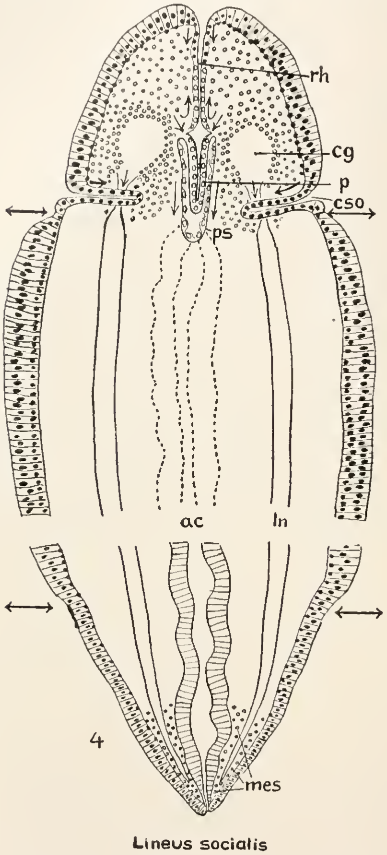
FIGS. 1–3, *Lineus socialis.*

Fig. 1. Diagram of regenerating fragment of body four days after operation, showing migration of epidermal and regenerative cells toward both cut surfaces as indicated by direction of arrows; *ac,* alimentary canal; *cgl,* cutis glands; *cm,* circular musculature; *ep,* epidermis; *ilm, olm, olm′,* inner and outer longitudinal musculatures, respectively; *ln,* lateral nerve cord. Double arrows indicate positions of cuts.

Fig. 2. Blastema preceding visible localization of organs; sixth day of regeneration; letters as in Fig. 1.

Fig. 3. Blastema showing localization of prospective cerebral ganglia (*cg*) and (in median line) proboscis and sheath; eighth day of regeneration; letters as in Fig. 1.

The regenerative cells which migrate anteriorly, on the contrary, form a true blastema consisting of an apparently undifferentiated mass of mesenchyme cells (Fig. 2). These cells form the basis of an essentially new individual. The blastema resembles the early embryo in that it seems to be a self-determining system, the new organs being differentiated without regard to the origin of their cells among the parent tissues. It is at present impossible to state which, if any, of the constituent cells of the blastema are derived from the new epidermis which covers the mesenchyme. All of the cells appear to be multipotent and capable of differentiation into any of future organs.



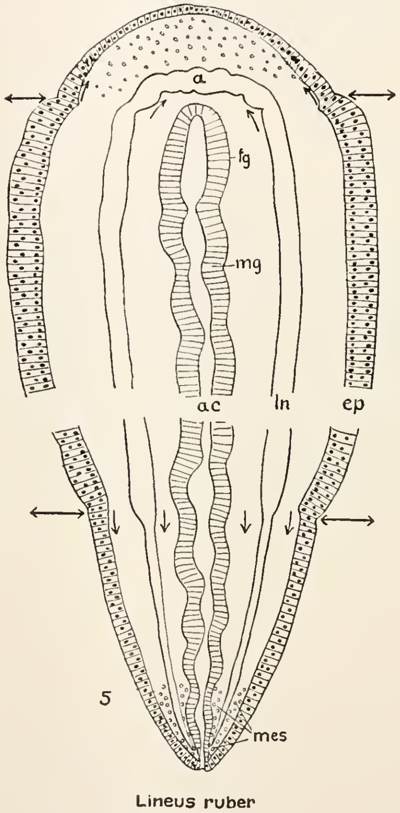


FIG. 4. *Lineus socialis.* Blastema with differentiated primordia of cerebral ganglia (*cg*) with new lateral nerves growing posteriorly to join nerve cords of original fragment; *cso*, invagination of new epidermis to form cerebral sense organs; *rh*, rhynchodeum; *p*, new proboscis growing posteriorly in the new sheath (*ps*). The posterior end shows the elongation of the original organ systems, with migration and incorporation of mesenchyme cells (*mes*). Fifteenth day of regeneration.

FIG. 5. *Lineus ruber.* Portion of body 30 days after operation, showing posterior regeneration only; wound healed at anterior end and nerve cords united, but no blastema is formed; letters as in Fig. 1; double arrows indicate positions of cuts.

Localizations soon become manifest in the blastema and the regenerative cells congregate in three principal areas (Fig. 3). Two of these are situated laterally and represent the primordia of the cerebral ganglia, while the median group furnishes the cells for the new proboscis and proboscis sheath.

The cells which are later differentiated into the ganglia are so closely associated with the basal cells of the new epidermis along the lateral margins of the blastema that it seems quite possible that some of them may be of epidermal origin. If so, they may be predetermined or partially determined in the direction of an epidermal derivative such as the nervous system.

Soon after their differentiation the primordia appear to act as induction centers for the other cephalic organs. From the base of the proboscis primordium a group of cells becomes differentiated into a slender tube of epithelium which represents the future rhynchodeum. Simultaneously a group of epidermal cells at the anterior margin of the blastema forms a slender invagination which joins the rhynchodeum. The proboscis is thus brought into communication with the exterior (Fig. 4).

The cerebral ganglia are likewise associated with, and presumably induce, a pair of epidermal invaginations on the lateral margins of the blastema. These represent the future canals of the cerebral sense organs (Fig. 4). The new mouth is formed by another epidermal invagination; this is evidently induced by the anterior end of the new foregut.

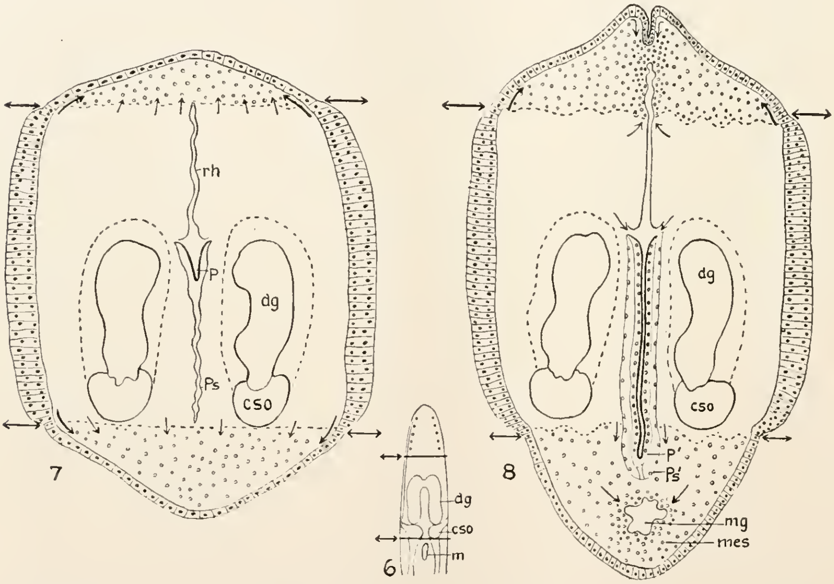
As soon as all the organs of the new head have been restored, that is, when the regenerated part has become individualized, it reorganizes all the tissues of the original fragment into units of smaller size. Nutrition by phagocytosis leads to compensatory growth of the new part, the size which is reached before external food is taken being obviously dependent upon the dimensions of the original fragment.

To accomplish this reorganization, the new organs, which have been differentiated in the blastema quite independently of the organs in the original fragment, grow posteriorly and make connections with such of the original organs as are represented in the fragment. The minute new nerve cords join the much larger cords of the fragment, the new mouth joins the old alimentary canal and the new blood vessels unite with the old. Phagocytic activities remove such parts of the old organs as cannot be incorporated into the new and transfer the available materials as nutrition for the new tissues. An essentially new individual of harmonious proportions results.

Since the original proboscis is not represented in the fragment the new proboscis, with its sheath, grows posteriorly into the parenchyma which fills the space formerly occupied by the old proboscis and sheath or in a corresponding position if the fragment was taken posterior to the end of the sheath. During this elongation the walls of the proboscis become differentiated into epithelial, muscular and connective tissue layers, as previously described (Coe, 1934). Nerves grow into the organ from the new brain.

Regeneration of a New Body from a Portion of the Head

The conditions are somewhat different when a new body is restored from the head alone or even from a portion of the head, as shown in Figs. 6 to 11. If two transverse cuts be made so as to remove the anterior portion of the head as well as the entire body posterior to the cerebral sense organs (Fig. 6), the tissues to be replaced anteriorly will be organized by those remaining. If all trace of the digestive system, including the buccal epithelium, has been removed from the posterior end of the head, an entirely new alimentary canal must be replaced. This is accomplished by mesenchyme cells which migrate posteriorly from the remaining cephalic tissues (Figs. 7, 8). These mesenchyme



Figs. 6–8. *Lineus socialis.* Early stages in regeneration of new body from portion of head, cut as indicated by double arrows in Fig. 6; *cso*, cerebral sense organ; *dg*, dorsal ganglion; *m*, mouth; *mes*, mesenchyme; *mg*, primordium of midgut; *P*, proboscis; *Ps*, proboscis sheath; *rh*, rhynchodeum; arrows show direction of migrating cells.

cells are preceded by wandering phagocytes which congregate at the site of the future midgut to form an irregular mass of cells with more or less tissue fluid between them. The mesenchyme cells then arrange themselves in a single layer around this fluid area to form the primordium of the midgut (Fig. 9). With further additions and by cell division the mesenchyme cells become differentiated into columnar epithelium characteristic of the digestive system (Coe, 1934).

A median outgrowth of the epithelium at the anterior end of the midgut forms the primordium of the foregut. This is followed by an invagination of the new epithelium on the ventral side of the body to form the new mouth (Fig. 10), exactly as in the case of the regenerating body fragments previously described. The foregut, of mesenchymal origin, then forms a communication with the buccal cavity, of epidermal origin, and the constituent cells are mingled.

These processes may be interpreted as implying that the epidermal invagination is induced by some stimulus originating in the newly formed foregut, since the epidermal cells appear to be activated only after the foregut has reached a definite stage of differentiation. The posterior end of the midgut is capable of indefinite extension, both by cell proliferation and by the incorporation of mesenchymal cells which are always present at the posterior end of the elongating body (Figs. 9–11).

Reorganization, or regulation, of the old cephalic tissues by phagocytosis supplies sufficient material for the minute new individual which results (Fig. 11).

REGENERATION IN OTHER SPECIES

In the genus *Lineus* are three groups of species which differ widely in their capacities for restoring new heads on fragments of the body (Coe, 1929, 1930, 1932, 1934). In one group, including *L. socialis*, discussed on the preceding pages, the ability to restore a new head from a body fragment extends the entire length of the body; in the second group, including *L. pictifrons*, this capacity extends only through the anterior half of the foregut region, while in the third group, of which *L. ruber* ("broad form," Nusbaum and Oxner) is an example, only that part containing the anterior ends of the nerve cords restores a missing head. If we inquire wherein these groups of species differ the answer may be found either in differences of distribution of the regenerative cells or in the agencies which may control their activities.

All three groups show similar capacities for wound healing and for posterior regeneration, and the parenchyma in all these species contains cells of similar appearance. In the first group, however, the cells which migrate anteriorly are immediately organized into the regenerative cells of the blastema and hence of an essentially new organism.

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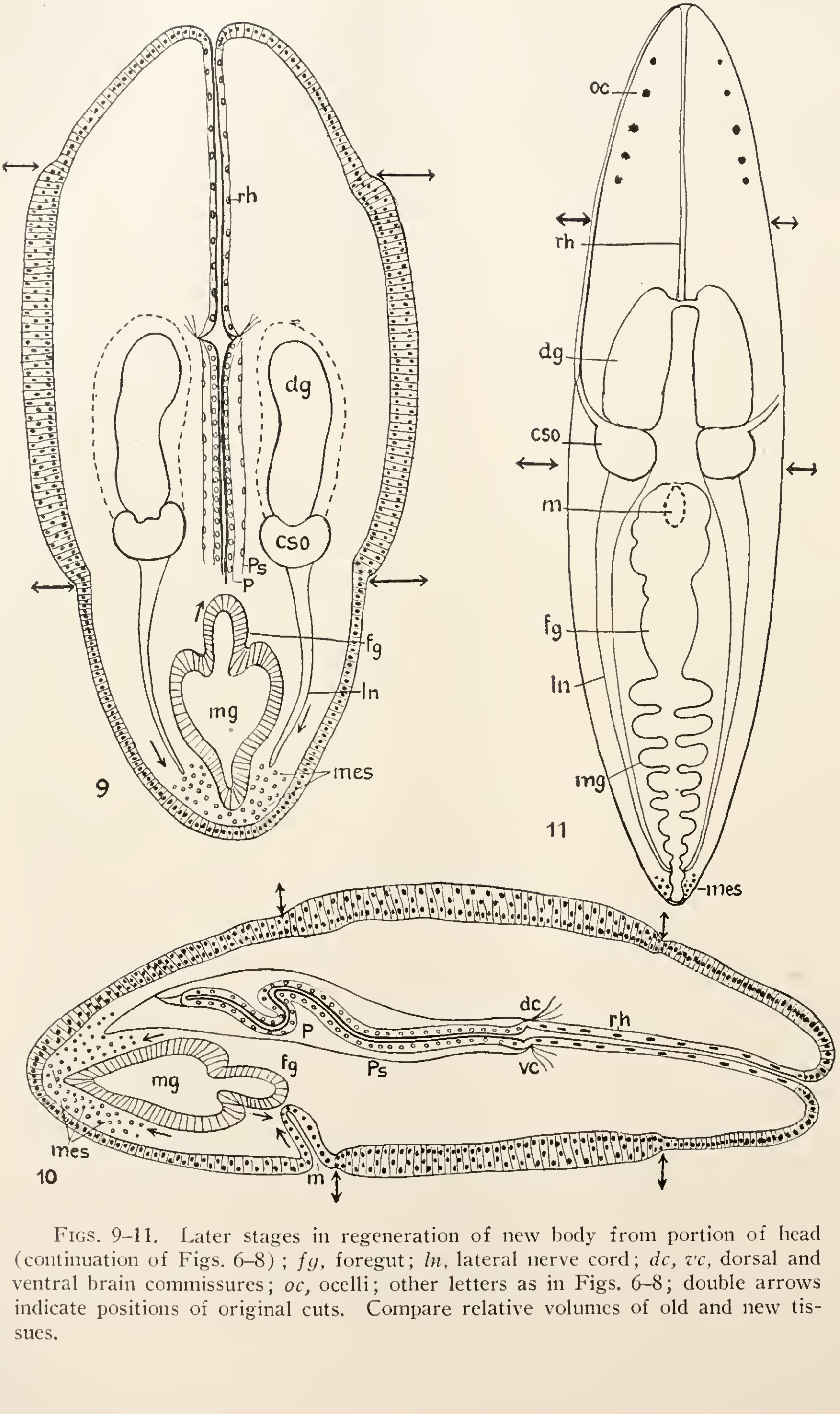
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FIGS. 9–11. Later stages in regeneration of new body from portion of head (continuation of Figs. 6–8); *fg*, foregut; *ln*, lateral nerve cord; *dc*, *vc*, dorsal and ventral brain commissures; *oc*, ocelli; other letters as in Figs. 6–8; double arrows indicate positions of original cuts. Compare relative volumes of old and new tissues.

In the third group, on the other hand, such cells as migrate anteriorly retain the characteristics of parenchyma cells and as such supply merely the connective tissues and phagocytic cells for the completion of wound healing but without restoring the missing parts. The growth of these connective tissues may be very extensive and the headless fragment may live for several months. During this period the organ systems may be considerably altered anteriorly, often leading to the fusion of the cut ends of the nerve cords in the median line (Fig. 5). But the old organism remains incomplete and eventually dies, presumably because it lacks either the cells necessary for the restoration of a new head or the suitable stimulus for their activation, or both. The possibility of the presence of an inhibiting influence must also be kept in mind. Since the head alone may restore a new body, while the body itself cannot replace the missing head, and since regeneration does not occur unless portions of the nerve cords are present, we may conclude that the organizing, or morphogenetic, agent is in these species limited to the head and more particularly to the anterior ends of the nerve cords.

The region of complete regeneration in any species would thus be coextensive with bipolarity in the movements of the regenerative cells, extending the entire length of the body in individuals of the first group but only to the posterior ends of the cerebral sense organs in the third. An intermediate condition exists in the second group, where this organizing potency extends from the brain to the middle of the foregut region.

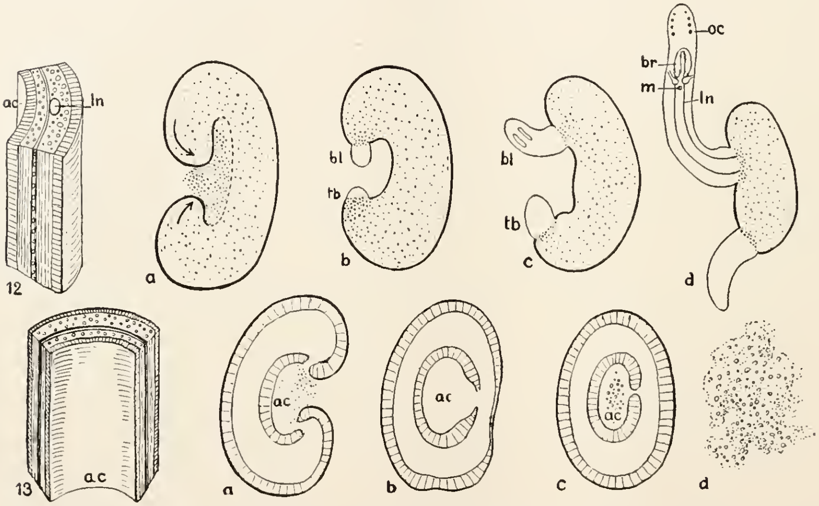
In the first group all parts of the body which contain any portion of the nerve cords are equally capable of regeneration and a hundred simultaneously regenerating fragments may be obtained from a single individual. In the second group only two new individuals have been obtained from one, while in the third group no increase in number has been found possible since only that fragment survives which contains the anterior ends of the nerve cords.

Corresponding differences in the regenerative capacities of different parts of the body in oligochætes are attributed by Stone (1932, 1933) to a restricted distribution of the regenerative cells, while Curtis and Schulze (1934) find that the regenerative ability in species of planarians is correlated with the relative number of regenerative cells preformed in the parenchyma.

Influence of the Nervous System

It must be kept in mind that the processes described appear to be in some way dependent upon the presence of some part of the nervous system. Repeated observation of body fragments of *Lineus socialis* indicates that at least a small portion of one of the lateral nerve cords must be present in order that complete regeneration and regulation may take place. Wound healing occurs normally in a fragment lacking any portion of the nerve cords and the remaining tissues may be reorganized extensively, but an anterior blastema is not formed. Such reorganized pieces may remain alive for several weeks but individualization is not accomplished and disintegration has followed in every case observed (Fig. 13).

Whether this lack of regenerative capacity is due to the removal of essential regenerative cells or whether to the absence of some stimulus required by the cells remaining is at present uncertain, but from a consideration of all aspects of the regenerative processes in this and other species the evidence seems to favor the latter alternative.



FIGS. 12, 13. *Lineus socialis.* Fig. 12, regeneration of sector of fragment containing portion of nerve cord (*ln*); *bl,* blastema at anterior end of nerve cord; *tb,* tail bud; *oc,* ocelli. Fig. 13, reorganization, without regeneration, of sector of fragment lacking any portion of nerve cord; disintegration after a survival of thirty days; *ac,* portion of alimentary canal.

The extent to which this stimulating agent is present along the length of the nerve cords in various species may, conceivably, be responsible for the observed differences in their regenerative capacities. For we must recall that in other, apparently closely related, species a new head is not formed even when the entire nervous system posterior to the cerebral sense organs is intact. Such differences in the regenerative capacities of morphologically similar species throws additional light on this problem of determination.

In fragments capable of regeneration, the blastema invariably makes its appearance at the anterior cut ends of the nerve cords. In fragments consisting of an entire transverse section of the body, the ends of the two nerve cords are brought closer together by the contractions of the musculatures near the cut surface, while in sectors of the body the single nerve cord ends in the recurved anterior end of the fragment (Fig. 12). In both cases the blastema arises in the new plane of symmetry (Figs. 1–3; 12) at the cut ends of the nerve cords because the regenerative cells have migrated to these positions.

In the planarians likewise the blastema usually arises near the anterior cut ends of the nerve cords, although in certain species even fragments cut lateral to the nerve cord show complete regeneration. In such cases, as Beyer and Child (1930) have shown, the head bud usually forms along the median border of the fragment adjacent to one of the lateral branches of the nerve cord or a group of such branches. They regard the central nervous system as an activating factor concerned in localization, and not as a determining factor nor as necessarily essential for the localization of anterior ends in the planarians studied.

There is a similar close connection between the cut ends of the nerve cords and the position of the regenerative buds in both polychæte and oligochæte annelids. As found by Goldfarb many years ago, removing the nerve cord from near the cut surface causes a considerable delay in the regeneration of earthworms (Siegmund, 1928; Kropp, 1933). If the ends of the cords are turned back and held in this position, normal regeneration does not take place (Bailey, 1930; Holmes, 1931). Evidently the migration of the regenerative cells to the cut surface is thereby prevented, although wound healing occurs as usual.

The inhibiting effects of X-rays on regenerative ability in various groups of animals appear to be due to the destruction of the regenerative cells or to changes which render them incapable of activation. Proliferation of the specialized tissues may also be checked, but wound healing is not prevented unless the dosage is very strong (Zhinkin, 1932; Stone, 1932, 1933).

The bilateral symmetry of the blastema in both planarians and nemerteans is not strictly dependent upon the symmetry of the original fragment, for a fragment taken from either the left or right side of the body produces a blastema of normal symmetry (Fig. 12). The migrating regenerative cells are evidently disposed with reference to the new plane of symmetry imposed by the group as a whole. An asymmetrical blastema has been found only when the original blastema has been split lengthwise. If the parts are then kept separated or one of them removed the original symmetry is slowly restored. Splitting the anterior end of the fragment before regeneration has made much progress causes the formation of a normal blastema on each part (Coe, 1930).

In the case of wound healing as well as in posterior regeneration the making of the cut causes a disturbance of the normal interrelations of the remaining cells, together with changes in their internal environment. The cells are thereby activated and respond adaptively to the new conditions, finally becoming organized into the functional tissues. The situation at the anterior cut surface is at first similar but complications soon arise because the fragment is without individualization. In addition to the necessity of repairing the organ systems represented in the fragment, a head with entirely new organs must be provided. Head formation resembles embryonic development in that the new organs become differentiated from groups of multipotent cells. In regeneration these cells are derived from dormant parenchyma cells which migrate forward to form the blastema as previously described.

In the activation of these cells it may be supposed that the cut nerve cords liberate some influence, not improbably a growth-stimulating substance, which acts specifically upon the dormant cells of the neighboring parenchyma, transforming them into active regenerative cells and directing their movements anteriorly.

Given this initial stimulus the multipotent cells arrange themselves or are arranged into an aggregate endowed with the power of self-determination. The differentiated primordia of the organ systems act as induction centers for the associated parts of the new systems, whereby the descendents of the original regenerative cells encounter one organizing factor after another in the series of regenerative processes discussed in the preceding portion of this paper. Each step in the series induces the one that is to follow as the organization of the essentially new individual proceeds.

SUMMARY

1. An attempt to analyze the organizing potencies in the regeneration of fragments of the body in several species of nemerteans leads to the conclusion that the cut nerve cords liberate an agent which activates the dormant cells of the parenchyma and transforms them into regenerative cells. Bipolar migration of these cells leads to complete regeneration.

2. The different regenerative capacities in closely related species may be dependent upon differences in the extent of distribution either of this activating agent or of the regenerative cells. In one group the entire length of the body is included and all parts are equally capable of regeneration; in another group the regenerative potency reaches only to the middle of the foregut region, while in a third group of species it is

limited to the anterior ends of the nerve cords and head-formation is limited to a single transverse plane.

3. The blastema is considered to be a self-determining system comparable to that of the early embryo. The constituent cells are evidently multipotent and capable of differentiation into any of the new organs. Once activated they and their descendants complete the regenerative processes.

4. The primary organization center is evidently associated with that part of the nerve cords which is capable of activating the regenerative cells and of controlling their bipolar migration. Secondary organization centers result as soon as the primordia of the organ systems have become differentiated in the self-determining blastema.

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(A more complete list of papers dealing with regeneration in nemerteans may be found in Coe, 1929, 1930, 1932, 1934.)

REGENERATION OF THE TAIL-FINS OF FUNDULUS EMBRYOS[1]

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That the fins of fishes possess the power of regeneration was first demonstrated by Broussonet (1786). From his experiments two observations are worthy of note in connection with this study. First he records that regeneration takes place more rapidly in young fish than in old and that the rate of regeneration may differ from one species to another. Secondly he states that the presence of the stumps of the fin-rays is necessary for regeneration and that in their absence regeneration will not take place.

Fraisse (1885) and Weismann (1892) state that there is very little power of regeneration in the fins of fishes. It is now a well-established fact, as pointed out by Morgan (1902), that many kinds of fish belonging to widely different families will regenerate their tail-fins.

The earliest recorded experiments on the tail-fins of fishes from the standpoint of morphogenesis were performed by Morgan (1900, 1902, and 1906). Later Morrill (1906), Scott (1907 and 1909), Beigel (1912), and Nabrit (1929 and 1931) performed experiments along this same line.

Broussonet (1786), Morgan (1906), and Morrill (1906) agreed that for regeneration to take place the ray stumps must be left. Morrill went so far as to suggest that regeneration does not take place even when the ray stumps are too small; on the other hand, Nabrit (1931) found that the ray stumps are not necessary for regeneration and that if the rays are entirely picked out new rays will appear under the influence of the articulating portion of the basal plate.

After comparing the findings of Harrison (1918) and Detwiler (1918) in *Amblystoma* with the results of his experiments on fish, Nabrit (1929) suggested that a possible similarity existed between the production of limbs in *Amblystoma* and of tail-fins in fishes. Both cases seem to be independently differentiating mesenchymal systems.

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[1] I am particularly indebted to Professor S. M. Nabrit of Atlanta University for suggesting this problem and to Professor J. W. Wilson of Brown University for his valuable assistance in the preparation of this paper.

The experiments which form the basis of this paper were planned for three purposes:

1. To examine further the relationship of the ray stumps to regeneration of the fin-rays.
2. To obtain, if possible, new data on the rôle of the basal plate in regeneration of the fin-rays.
3. To compare the regeneration process in the embryo with facts already recorded for the adults.

These experiments were started at the Biological Laboratory at Cold Spring Harbor in the summer of 1931 and were completed there in the summer of 1933. Fixed preparations of the material were studied at Arnold Biological Laboratory, Brown University.

Material and Methods

The material chosen for these experiments was embryos of *Fundulus heteroclitus*. These embryos were obtained from the eggs before the time of hatching by the method devised by Nicholas (1927). The eggs were permitted to develop until the desired stage was reached and were then removed from the chorion. Great care was taken in this removal because the slightest pressure exerted on the eggs will cause injury to the embryo or yolk sac which will render the animal unfit for experimental purposes.

Embryos which are injured show the effect very soon after their removal from the chorion. After a few hours to see if the embryos had been injured in their removal they were ready for operation. Because of their small size the embryos must be operated on under the microscope and therefore it was necessary to employ a method whereby small bits of tissue might be removed. For this purpose Nicholas' (1927) modification of Spemann's technique was used. The cut was made by drawing the tissue to be removed into the lumen of a very fine pipette and carefully cutting the tissue against the tip of the pipette with a spear-point blade. In some cases, especially in embryos just about to hatch, it was found best to remove the parts by supporting the embryo with an ordinary dissecting needle and making the cut with a spear-point blade. In either case great care must be taken not to press the embryo against the bottom of the dish as this invariably results in death. The embryos lack movement and therefore anesthesia is unnecessary. In order to stop the bleeding which occurs when the cuts were made well up into the body, it was found best to transfer the operated embryos immediately to cold sea water or to a cold sodium chloride solution that was isotonic with sea water. The latter was found to be more satisfactory.

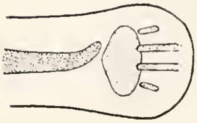
Throughout the course of the experiment the embryos were kept in small dishes and the water changed twice daily. No attempt was made to control the temperature as during the summer it was fairly constant in the laboratory, varying only from 18° to 21° C.

Observations were made mainly on living specimens which were placed in a small drop of water on depression slides and examined under a microscope with a 32 mm. objective. A few individuals were fixed in Bouin's fluid and preserved in alcohol for microscopic examination. These were stained in toto with alizarin and cleared in oil of wintergreen. Camera lucida was employed for recording the regeneration process.

EXPERIMENTAL

Preparatory to experimentation a study was made of the normal process of formation of the tail-fins and of the articulating plate. Nabrit (1929) describes the development of the tail-fins as follows: (1) there is a primitive natatory fold; (2) the mesenchymal mass from which the fin-rays and articulating plate develop forms between the end of the notochord and the extremity of the fold; (3) from this mesenchymal mass central rays are the first to differentiate; (4) at the time of differentiation of the first rays the natatory fold is further from the base of the notochord in the central region than in the dorsal and ventral regions; (5) in individuals that hatched in sixteen days the streaking of the rays begins on the seventh day; (6) the additional rays are added dorsally and ventrally but the blood vessels that pass between the rays may be seen to loop in the paths of the rays before the rays are vitally stainable with Nile blue sulphate or with alizarin after fixation. That the rays are present before they can be seen in stained preparations may be demonstrated by crushing the tail under a coverslip and observing the process of fragmentation. By the time the anlage of the first rays to develop are stainable, they are no longer connected with the mass which develops into the basal plate but form an articulation with it (Fig. 1). While my observations confirm Nabrit's in general, there is some doubt as to whether or not the rays and articulating plate develop from the same mesenchymal mass. Soon after hatching the most caudal of the vertebral spines enlarges and becomes incorporated in the plate (Fig. 2). Later the next spine anteriorly does the same and this process is continued until the adult pattern of the tail is formed (Fig. 3).

The experimental animals are divided into three groups dependent upon the mode of operation employed: (1) splitting of the tail-bud; (2) removal of the entire tail; (3) removal of part of the tail.



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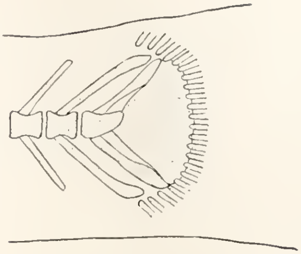

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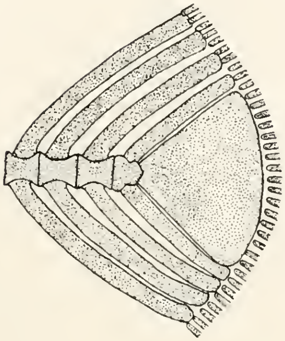

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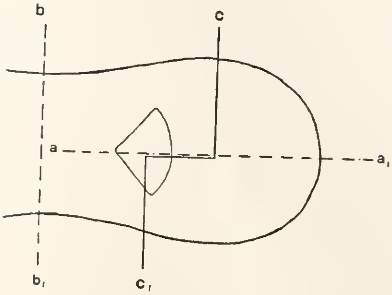

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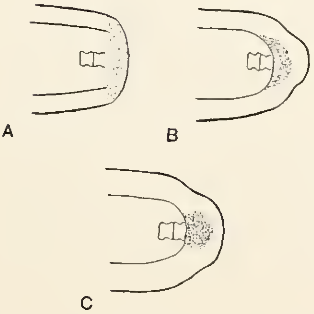

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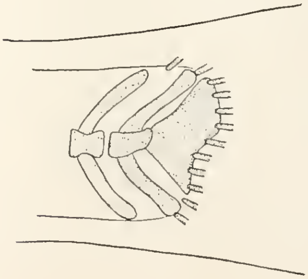

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Fig. 1. Diagrammatic sketch of the tail-fin anlage just prior to the time it becomes stainable. The largest rays are the first to separate from the mass and articulate with the plate.

Fig. 2. A camera lucida drawing of the tail at the stage of development when the most posterior of the vertebral spines become incorporated in the plate. (Four days after hatching.)

Fig. 3. A camera lucida drawing of the basal plate of an adult showing the relationship of the vertebral spines to the articulating border of the plate.

Fig. 4. Diagrammatic sketch showing the relationship between the basal plate and the type of cuts made in the various experiments.

Fig. 5. Camera lucida drawings made at various stages after removal of the entire tail-fin anlage. (*A*) twenty-four hours after operation; (*B*) six days; (*C*) twenty-five days. See text for explanation.

Fig. 6. A camera lucida drawing showing the articulation of the regenerated rays with the exposed edge of the basal plate in the ventral region of the tail.

1. The tail-fins of embryos from seven to fifteen days old were deeply incised as nearly as possible through the middle of the fins and basal plate in a posterio-anterior direction, the cut being made along the line a–a_1 of Fig. 4. Such an incision divided the fin, the plate, and a portion of the posterior part of the body into dorsal and ventral parts. The operation is of such a drastic nature, so long an incision being made, that many of the animals operated upon in this manner died soon after the cut was made. The survivors were kept from ten to twenty days and at the end of this time all of the cut plates appeared to have fused together, though in some cases development of the tail was somewhat retarded. The wound usually left a distinct scar or groove running across the tail-bud, which, however, was obliterated after several days. In no case did the operation result in reduplication.

2. Tails were removed from embryos in all stages of development from the fifth day, at which time there is no visible differentiation of the fins or plate, until the time of hatching. The cut was made just anterior to the region of the basal plate and never as far anterior as the position of the dorsal and ventral fins, the cut being made along the line b–b_1 of Fig. 4. In about twenty-four hours after operation the wound heals and by this time there appeared some darkly staining tissue at the level of the cut (Fig. 5, A). By the sixth day this darkly staining tissue had rounded into a small nodule and had taken a place at the end of the spinal column (Fig. 5, B) where it remained as long as the fish was allowed to live without showing any tendency to undergo further differentiation. The mass at the end of twenty-five days is shown (Fig. 5, C) and is the same in appearance as on the sixth day.

In none of the embryos operated on in the above manner did any signs of regeneration of the plate or fins appear up to and including the sixty-fifth day after operation. The stage of development at which the operation is performed seems to have no bearing on the results, in all cases the process was apparently the same. It is doubtful whether regeneration of the plate or fins would ever occur after this type of operation but at present it can only be stated that regeneration fails to occur in the allotted time. Some experiments were carried out on young hatched *Fundulus* of unknown age (measuring from one to two centimeters in length) and the same results were obtained as above with the exception that no nodule was formed at the end of the spinal column. These older animals did not survive as well as the embryos, for death usually ended the experiment between the twentieth and thirtieth day after operation. The cause of death of these fish was apparently a sloughing-off of the tissue starting at the level of the cut, this sloughing process proceeding anteriorly until death took place.

3. Embryos twelve to fifteen days old, at which time the rays and plate are well differentiated, were operated upon so as to remove the ventral half of the plate and all of the rays distal to it, while in the dorsal half of the tail only the distal half of the rays were removed, the cut being made along the line c–c_1 of Fig. 4. At this time there were from six to ten rays articulating with the basal plate. The animals were allowed to regenerate and at the end of from fifteen to seventeen days some were fixed and stained with alizarin. In the ventral half of the tail a portion of the basal plate had been removed prior to differentiation of some of the rays and there was also removed a part of the basal plate with which some rays were articulating. Under the influence of the exposed edge of the basal plate, rays differentiated in the mass of regenerating tissue. These rays were similar in every respect to the other rays except for their more anterior articulation. These new rays articulate with the exposed edge of the basal plate (Fig. 6). This is true regardless of whether the part of the plate they articulate with is derived from spines or from the original central plate. In the latter case rays come out from the border of the plate even though it is not at the level of the unoperated portion of the plate. The shape of the plate may in this way influence the shape of the tail because, after reaching a definite size, the rays cease to grow in length and the tail remains somewhat shorter on the operated side. Regeneration of the plate is a relatively slow process as compared with the rays and in the length of time that the majority of the animals were under observation, usually seventeen days, the plate had not returned to its original size or shape (Fig. 7). In a much longer period, namely fifty days, it was found that the plate had almost completely replaced itself and at this time the tail ceased to be asymmetrical. So far as general observation reveals, the developmental processes of all other parts of the fish are normal.

DISCUSSION

Concerning the extent of regeneration that will take place in the tails of fishes there seems to be, according to the literature, quite a diversity of opinions. All of the papers concerning this phase of the problem cannot be discussed but only the more outstanding ones will be mentioned.

Nussbaum and Sidoriak (1900) have demonstrated that *Salmo fario* operated on the day after hatching would regenerate the posterior part of the body in about ten weeks. If the cuts were made anterior to the anus a new posterior opening is established as well as a new opening for the urethra. After ten weeks fungus usually caused death of the culture.

Duncker (1905), using *Syngnathus*, demonstrated that when the entire tail is removed a perfectly normal one is regenerated. Also, if part of the vertebral column is cut out, accessory tails are produced at the point of injury. However, he was unable to obtain the same results on any other forms used.

The difference between the results obtained from the experiments recorded in this paper for *Fundulus* embryos and those recorded by the above investigators for *Salmo fario* and *Syngnathus* is not apparent at the present time but suggests an explanation on the basis that different species show quite different powers of regeneration.

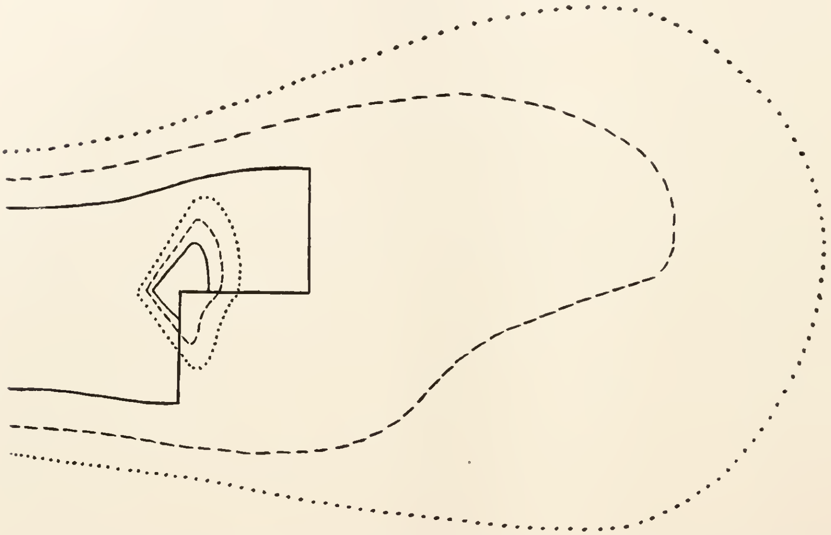

FIG. 7. A composite diagrammatic sketch showing the relative amounts of plate and fin material replaced at different stages after operation. The solid line represents twenty-four hours, the broken line twenty days and the dotted line thirty days after operation.

In a *Fundulus heteroclitus* embryo which was cauterized just posterior to the body cavity prior to tail-fin differentiation, Nabrit (unpublished results) has obtained an abnormal tail with four rays. The fish died soon after hatching. It was not possible to determine the exact origin of these rays. They appeared, however, in the center of the posterior surface. The fish did not regenerate the lost part of its body. There is no definite indication that regeneration will ever take place after such an operation but rather the undifferentiated mesenchymal bud of the dorsal and ventral fins may differentiate at the end of the animal and simulate a tail.

The observations recorded in this paper on removal of the entire tail-fin anlage indicate that the factors responsible for regeneration do not extend more anterior than the region of the basal plate. The experiments on removal of parts of the plate reveal that the rays can be regenerated even before the plate has completed itself. These results indicate that the regenerative factors, be they nervous system, distance from the base of the tail, axial gradient of metabolism, or other factors, are segregated in the plate-fin region early in the process of differentiation. In fact, this type of segregation apparently takes place before any visible differentiation has taken place. This observation confirms the work of Nicholas (1927), who has shown that *Fundulus* embryos undergo early in their development an extremely high degree of differentiation. Such a fact in itself may explain in part the lack of power of regeneration of certain parts. That splitting of the tail does not result in reduplication is a result to be expected considering the extremely high degree of differentiation in these embryos. Such results as reported above are not confined to *Fundulus* embryos as similar results have recently been obtained by using young sunfish and guppies.

It has been demonstrated that if all of the ray material and part of the basal plate is removed, the basal plate will replace itself and that rays will be differentiated in the regenerating tissue under the influence of the exposed edge of the basal plate. This fact indicates conclusively that the presence of ray stumps is not necessary for regeneration of the rays to take place.

Harrison (1918) and Detwiler (1918) have shown that in *Amblystoma* the fore-limb develops from a self-differentiating and equipotential system. The limb anlage therefore is an entity, which, except for its dependence for nourishment, is independent of its surroundings in the attainment of specific form. Detwiler showed that it was possible to initiate the development of a limb in almost complete absence of the shoulder girdle. They suggest that the limb anlage may be regarded not as a definitely circumscribed area, like a stone in a mosaic, but as a center of differentiation in which the intensity of the process diminishes as the distance from the center increases until it passes away into indifferent regions. This conception suggests that the growth of the limb may be controlled by the distance of the part from the center of the anlage. If the anlage is extirpated other mesenchyme could, however, in a restricted sense, simulate the extirpated anlage and produce an apparently normal limb. This explanation is based on the conception of the mesenchyme as a formative factor in growth and, according to Nabrit (1929), the same explanation may be offered for the type of regeneration obtained in the tails of fishes.

It is concluded, therefore, that regeneration of the tail-fins of fishes and the same process in the limbs of *Amblystoma* are similar. The basal plate is apparently like the shoulder girdle in regenerative capacity, since it may replace itself and give rise to or induce the development of rays. Hence the rays, like the limbs of *Amblystoma,* and the basal plate, like the girdle, belong to a self-differentiating mesenchymal system.

SUMMARY

1. A study was made of regeneration of the tail-fins and basal plate of *Fundulus heteroclitus* embryos operated upon prior to the time of hatching.
2. Splitting of the tail-fin anlage does not result in reduplication of the tails.
3. Removal of the entire tail-fin anlage results in no regeneration up to and including the sixty-fifth day after operation.
4. If part of the basal plate is removed with the rays distal to it new rays will appear in seventeen days and the plate will regenerate in fifty days.

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PRESENT STATUS OF LONGITUDINAL DIVISION IN HYDRA

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Longitudinal division in *Hydra* has long been considered a method of reproduction, but it was admitted that it was rare in occurrence. The author wishes to point out works which show that the process is merely one of regulation and not of reproduction, and to substantiate this view with some of his own experience.

Several authors have described longitudinal division, Trembley (1744) probably being the first; but it should be noted that Baker, working at the same time as the Swiss priest, also noted this phenomenon. Jennings (1883) records a similar process as do also Koelitz (1909) and Ross (1914). These last three authors have merely described the process of division and have not entered greatly into discussion of it. Trembley noted that the division could be instigated by cutting the oral end of the animal.

Marshall (1882) found that if the anterior end of a hydra were partially split in two, each half became a distinct anterior end. The body then began to separate slowly into two parts, the division taking place at the angle between the two oral ends until the two parts became completely separated. King (1901) repeated the experiment in a large number of cases with practically the same result. The work of these men and others shows beyond a doubt that hydra can be stimulated to divide longitudinally.

Morgan, in his memorable book on regeneration, quotes von Kennel as asking the questions, " Can accidental injuries account for the result (viz. for the division in *Lumbriculus,* planarians, and starfish), since how few starfish are there with regenerating arms in comparison with the enormous number of uninjured individuals? Should we not rather look for the external stimuli that have initiated the process of self-division?"

Morgan gives his own opinion on the subject when he says, " Hydra appear rarely, if at all, to divide by a cross-division, and, although one or two cases of longitudinal division have been described, it is not improbable that they have been started by the accidental splitting of the oral end." More recently Hegner (1931) states that the longitudinal division is the result of the animal's readjustment to release itself from an abnormal condition.

During the writer's recent work on regeneration (Roudabush, 1933), it was his privilege to make a series of observations which will lend some evidence toward the views of von Kennel, Morgan and Hegner. It was noted that during the process of turning hydra inside out, a number of individuals were torn at the anterior end, and since these were not useful for the problem then at hand, they were isolated in other culture dishes and examined at intervals to note just what would be the outcome of such injury. A large number of these injured specimens regenerated into normal individuals while a smaller percentage was found to develop two anterior ends and eventually complete the division. Thus a large number of dividing specimens was seen and all were begun by some injury inflicted on the anterior end. No notes were kept on these discarded animals, so no relationship can be definitely drawn between the number injured and the number showing division.

More recently a good stock of animals was secured for the purpose of obtaining a percentage relationship between those which showed division and those which did not. Both *Pelmatohydra oligactis* (Pallas) and *Hydra vulgaris* Pallas were used in this experiment. The animals were cut, or rather torn, through the mouth to a distance just posterior to the base of the tentacles. The distance was practically the same for every specimen so as to eliminate the possibility that some would have greater stimulus than others to divide. These animals, after having been cut, were placed in small dishes and examined daily until all the apparent injuries had healed. Those with two oral ends and slightly divided bodies were judged to show longitudinal division. These criteria were chosen merely because if such a condition were found in nature the specimen would be promptly judged to be undergoing division. The author realizes that some of these may never have completed the division either because of depression ensuing or because of the absorption of one of the anterior ends, but it still remains true that such specimens if found would have been considered to show division.

Sixteen and nine-tenths per cent of the animals treated as described showed the evidence of division. This percentage, doubtless, would have been much higher had the animals been torn farther down the body. (It should be noted that 11 per cent of the hydras which did not show division had an increase in the number of tentacles; most of them increasing only by one, but several by two.) This percentage may vary with hydras taken from different conditions because their ability to regenerate depends upon their past history. The fact that the percentage is low does not affect its significance.

The literature itself is evidence to the fact that longitudinal division occurs very rarely or at least it is rarely recorded. The facts discussed above show why this should be. If a number of hydras should happen to become injured at their anterior ends, only about 17 per cent of this number would regenerate two oral ends and undergo division. Of this 17 per cent, only a very few would ever fall into the hands of a scientific observer and so the number of records necessarily would be low. These records would also be lowered if the number of injured hydras were by any means kept low.

The foregoing statements are offered as an answer to von Kennel's first question. As to his second, let us consider how hydras could become injured in nature in such a manner as to cause division.

Since it is the mouth which is primarily involved, is it not conceivable that, should the animal attempt to take in pieces of food—worms or crustaceans—which are too large, this act would tear its mouth and thus instigate—at least in some cases—the division?

The author has seen such activity both in aquaria and in animals taken from their native pools. One case is of particular interest since it was watched through nearly the entire process.

While examining newly caught hydras one day, it was noted that one was attempting to swallow a worm which was nearly as large as the hydra itself. The worm, needless to say, was still struggling even though it was half inside the hydra. The worm was pushing against the side of the mouth of the hydra, as if in an effort to pry itself free. Other duties made it necessary to set these animals aside for a short time and when they were again observed the worm had in some manner released itself and in the process had split the hydra through the mouth. As the observation continued, the hydra developed two oral ends and showed typically longitudinal division as described above.

Since this answers von Kennel's question, and shows that the division can be caused by an external stimulus rather than by an internal condition, it is highly probable that it is a process of regeneration or regulation rather than one of reproduction.

SUMMARY

1. It is concluded that longitudinal division in *Hydra* is not a reproductive process but is one of regeneration.

2. Longitudinal division is stimulated by the tearing of the hydra's mouth.

3. About 17 per cent of those animals torn undergo division; the remainder merely heal the cut.

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THE INFLUENCE OF TEMPERATURE UPON THE AMYLASES OF COLD- AND WARM-BLOODED ANIMALS

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The temperature relations of the enzymes of cold-blooded animals are a subject of considerable interest. Apparently these enzymes act most efficaciously at temperatures seldom or never attained by the living animals. It has not been established irrefragably that the enzymes of the poikilotherms are adapted to act at lower temperatures, *i.e.* that the temperature coefficient is so small as to make the enzyme about as effective at low temperatures as at high.

The best way to study the relation between temperature and the activity of an enzyme is to study the temperature coefficients over a wide range, with rigid control of as many factors as possible. This has not been done adequately for representative animals of different vertebrate classes. A great many of the experiments which have been done have been carried on without any regard for pH, ionic concentrations, digestion periods, or enzyme-substrate ratios. As Haldane (1930) remarks of temperature coefficients of enzymes generally, " Many of the data in the literature are clearly unreliable." Kendall and Sherman (1910) showed how little a temperature coefficient may mean if the experimental conditions are not regulated properly; in the absence of electrolytes, they found the optimal temperature for amylase to be 20° C., but with added NaCl, the activity at 40° C. is four times as great as at 20° C.

It is the purpose of the present investigation to compare the temperature relations of amylases of representative cold- and warm-blooded animals. The temperature coefficients for the amylases were determined between 3.5° and 65° C., with rigid control of the factors just mentioned. These data should answer the questions of whether the amylase of the cold-blooded animal is adapted to act at lower temperatures, and if it has a lower optimal temperature than the amylase of the warm-blooded animal.

Incidentally, data will be presented which show that the " optimal temperature" is influenced by the period of digestion and by the enzyme-substrate ratio.

In Table I is given a summary of some of the literature bearing upon the problem of temperature adaptations of cold-blooded animal enzymes. For the reasons given above, many of the recorded results can not be accepted as valid and consequently many papers are not cited. [The literature up to the beginning of the present century is reviewed by Yung (1899); Sullivan (1907) provides an English equivalent.]

It is of interest to compare the temperature coefficients found for amylases of different origins with those to be reported here. The literature is tabulated in Table II; the coefficients were calculated from the data given, as in most cases the Q_{10} was not given by the authors.

TABLE I

Temperature adaptations reported for digestive enzymes of cold-blooded vertebrates

Investigator	Date	Source and enzyme	Adapted to lower temp.	Lower optimum than mammalian
Riddle..............	1909	Fish, amphibian and reptilian pepsin	Yes	—
Rakoczy...........	1913	Pike pepsin	Yes	Yes
Hosaka.............	1918	Frog amylase and trypsin	Yes	Yes
Müller..............	1922	Frog pepsin	No	No
Kenyon............	1925	Pickerel pepsin	Yes	Yes
		Fish, amphibian and reptilian pepsin	No	No
Oshima and Sasaki...	1925	Fish pepsin	Yes	Yes
Oya and Harada.....	1926	Fish amylase	—	No
Yio.................	1931	Fish amylase	Yes	—
Pjatnitzkij..........	1931	Frog pepsin	No	No

MATERIAL AND METHODS

The amylases of human saliva, terrapin pancreas, and fish pancreas were studied, using the copper reduction method described by Mathews (1920) and two iodine methods detailed below. All three methods were used simultaneously. The temperatures at which the experiments were made were: 3.5 ± 1°, 15 ± 1°, 25°, 35°, 45°, and 63° C.—the last four being constant to 0.05° C. Eight to ten experiments were made with each enzyme.

Preparation of Enzyme Solutions

Human Salivary Amylase.—After washing the mouth several times with water, the flow of saliva was stimulated by chewing on paraffin.

The saliva was collected in a beaker. To facilitate accuracy and rapidity in pipetting, the saliva was diluted with seven volumes of distilled water; it was used at once.

Reptilian Amylase.—The amylases of two reptiles were used: *Terrapene carolina* (Linnæus) and *Chrysemys* sp. The pancreas was removed from the freshly killed animal, rinsed, blotted and ground with glass fragments. The mass was suspended in 50 per cent alcohol for twenty-four hours. The alcohol-tissue ratio was adjusted to give approximately the same amylase concentration as was found in the diluted saliva, *as measured at 35° C.*

TABLE II

The temperature coefficients for amylases, recorded in literature

Source	Q_{10}						Author
	0–10°	10–20°	20–30°	30–40°	40–50°	50–60°	
Beef pancreas......	3.3*	2.25	1.6	1.0	0.7	0.17	Roberts (1881)
Malt.............	2.8	1.9	1.6	1.6	—	—	Müller-Thurgau (1885)
Malt.............	—	—	1.9	1.6	1.3	0.6	Vernon (1901)
Beef (?) pancreas...	—	—	2.0	2.0	—	—	Kendall and Sherman (1910)
Malt.............	—	—	2.2	1.4	—	—	Van Laer (1912)
Potato............	—	1.5	1.05	1.04	0.5	0.6	Doby (1914)
Merck's diastase...	No temperature effect between 5° and 45° C. Experiments at 5° intervals.						Koenig (1920)
Malt.............	—	—	1.96	1.65	1.43	—	Lüers and Wasmund (1921)
Human saliva......	2.9*	2.2	1.9	1.6	1.2	—	Ernström (1922)
Beef (?) pancreas...	—	—	2.1	1.8	1.4	0.77	Cook (1925)
Malt.............	—	—	1.8	1.6	1.3	0.76	" "

* Simple extrapolation.

Fish amylase.—The pyloric cæca and interdigitated pancreatic tissue of the menhaden, *Brevoortia tyrannus* (Latrobe), were removed and treated exactly as was the pancreas of the terrapin.

Preparation of the Digest

A 2.5 per cent preparation of soluble starch served as substrate. Phosphate buffers, final concentration 0.05 M, maintained pH 6.9. Ernström (1922) has shown that the factor *A*, of Arhennius' equation, and therefore the temperature coefficient, is independent of pH in the case of amylase. Hence the results with different amylases are comparable even though pH 6.9 may not be optimal for all. Sodium chloride was added to give a final concentration of 0.05 M. The final concentration of ethyl alcohol was about 0.22 M (1 per cent). In the fish and reptilian enzyme experiments, the alcohol served as the menstruum for the enzyme; and for the sake of uniformity of conditions, it was added in the determinations with salivary amylase. Of course there is a difference in that the salivary amylase had not been exposed to alcohol for twenty-four hours.

Fifty-milliliter portions of the soluble starch preparation were put into Ehrlenmeyer flasks and allowed to come to the temperature of the respective baths. At ten-second intervals, one milliliter of enzyme solution was added to each of the series in turn. The flasks were shaken to ensure thorough mixture.

Determination of Activity

The Iodine Methods.—The first iodine method consisted in determining the time required for the digestion of the soluble starch to the point at which it just fails to give the blue color reaction with iodine; the end-point is that described by Wohlgemuth (1908). At intervals of one minute, five drops were removed from the digest and added to 5 ml. N/8,000 iodine solution. (See Johnson, 1908; Chesley, 1931 and 1934.)

The second iodine method consisted merely in continuing the above procedure to determine the time in which no color reaction occurs with iodine. The methods will be referred to as " no blue " and " achromic " iodine methods.

The Copper Reduction Method.—At the end of thirty minutes, a 5 ml. specimen was removed from each of the digests, described above, and run into an excess of Fehling's solution (25 ml. diluted to 95 ml. with distilled water). The mixture was put into a bath at 112° C. and left for exactly 6 minutes. It was then removed and filtered through a Gooch crucible, using suction. The precipitate was washed with distilled water at 60° C., then dissolved in M/2 ferric ammonium sulphate-sulphuric acid mixture and immediately titrated with N/2 potassium permanganate. The amount of reducing sugars can then be calculated from this titration value, and the enzyme activity thus estimated.

Results and Discussion

The Temperature Relations of the Amylases

When the activities of the enzymes are plotted against temperature, very similar curves are obtained for all three amylases, whatever the

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method of determination used. (See Fig. 1. From Table III, it is evident that the copper reduction method gives curves similar to those obtained with the iodine methods.)

From the divergent courses of the curves above 35° C., it may be seen that the optimal temperatures for the same period of time are somewhat lower for the cold-blooded animal enzymes than for human amylase. This is, of course, due to heat destruction of the enzyme.

TABLE III

The temperature coefficients for the amylases of the human being, terrapin and menhaden. At 45° C.—"no blue," 10 minutes; "achromic," 25 minutes, digestion. At all temperatures, copper reduction, 30 minutes.

Range	Human salivary amylase			
	No blue	Achromic	Cu. red.	Mean
°C.				
5–15*	2.4	—	—	—
15–25	1.8	1.9	2.8	2.1
25–35	1.7	1.6	1.5	1.6
35–45	1.7	1.6	1.2	1.5
45–63	1.3	1.0	0.9	1.1
	Terrapin pancreatic amylase			
5–15*	3.3	3.4	3.2	3.3
15–25	2.0	2.0	1.8	1.9
25–35	1.6	1.5	1.2	1.4
35–45	1.6	1.6	1.5	1.5
45–63	0.6	—	0.7	0.6
	Menhaden pancreatic amylase			
5–15*	2.7	2.5	3.3	2.8
15–25	2.2	2.0	1.7	2.0
25–35	1.6	1.5	1.3	1.5
35–45	1.0	0.6	0.9	0.8
45–63	—	—	—	—

* Simple interpolation.

From the relative positions of the curves for the "no blue" and "achromic" methods, it is evident that the order of heat inactivation of the amylases is fish> terrapin> human. However, this difference in the behavior of the crude enzyme preparations does not justify the statement often made that the amylases are different. The heat inactivation of the enzyme is probably due to protein coagulation; the proteins generally of poikilothermal animals have lower coagulation temperatures than those of warm-blooded animals. The enzyme may be dragged down bodily by concomitant proteins while actually little affected by the heat. Also amyloclastic activity may be due to some specific chemical group prosthetic on, or inherent in, the protein molecule and common to amylases of all origins. Yet the behavior of the enzyme would be conditioned by the behavior of the whole complex.

From the origins of the curves, and from the temperature coefficients between 5° and 15° C., it may be seen that the amylase of the menhaden is better adapted and that of the terrapin less adapted to act at lower temperatures than is that of human saliva. In the matter of temperature adaptation, it would be interesting to study the enzymes of arctic fishes feeding actively at temperatures near 0° C. Probably the temperature coefficients between 0° and 10° C. are low for such enzymes; this is suggested by the coefficient for menhaden amylase in this range.

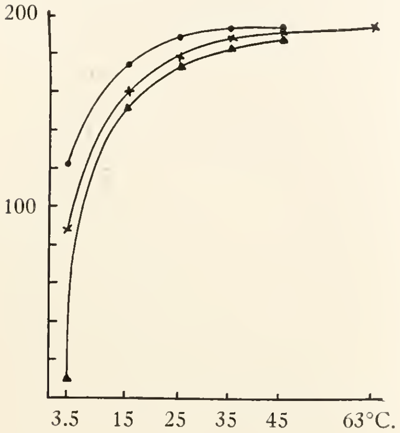

FIG. 1

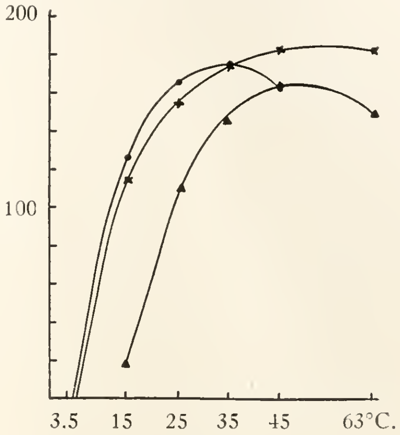

FIG. 2

1. "No blue" method.
2. "Achromic" method. The influence of temperature upon the activities of amylases of cold- and warm-blooded animals. Ordinates: (200–T). T is the time required for the disappearance of the color reaction with iodine. x indicates human salivary amylase; △ terrapin pancreatic amylase; ● menhaden pancreatic amylase.

The temperature coefficients for the different amylases studied have been calculated and tabulated in Table III. Between 15° and 35°, the coefficients are substantially the same for the three amylases. The variations below and above this range have been discussed above.

The intermediate position of human amylase, in adaptation to low temperature, is of interest in that it does not accord with Riddle's (1909) finding for pepsin. Riddle concluded, from his experiments upon digestion in Mett's tubes introduced into the stomachs, that "higher forms" progressively lose the power of digestion at lower temperatures. Thus he thought that he had demonstrated an evolutionary series in the development of digestive power.

Factors Influencing the "Optimal Temperature"

A comparison of the curves (Figs. 1 and 2) for any one of the enzymes, obtained by the different methods, shows that the longer the period of digestion the lower the optimal temperature. The "no blue" method covers the least time—ten minutes at 45° C. The optimum, as determined by this method, is about 40° C. for menhaden amylase. The achromic method gives 35° as the optimum for the same enzyme acting in the same digest for an additional fifteen minutes (as determined at 45° C.).

The enzyme-substrate ratio is another factor which will cause the optimal temperature to vary from experiment to experiment unless a constant proportion is set. In the experiments with saliva, the achromic point was reached first in the digest at 63° when the usual concentration of enzyme was used. With half this concentration, the digest at 63° lagged behind those at 45° and 35°. With still less enzyme, the 63° digest never reached the achromic end-point, due to heat inactivation of the enzyme; yet of the series, this was the first to reach the "no blue" end-point. This fact is, of course, a corollary of the fact that the optimal temperature varies with the period of digestion.

It is almost meaningless to speak of the "optimal temperature" of an enzyme without specifying pH, ionic concentrations, period of digestion and enzyme-substrate ratio. This cumbersome changeling will still be without any great significance except to give some idea of the thermostability of the enzyme. While the optimal temperature of an enzyme may be relatively high if the digestion period is short, the optimum will be considerably reduced as the period is prolonged.

SUMMARY AND CONCLUSIONS

The temperature coefficients for the amylases of human saliva, terrapin and fish pancreas have been determined for the range between 3.5° and 63° C.

Three methods were used simultaneously; a copper reduction method and two iodine methods.

Certain factors were found to influence the temperature effect upon the activities of the amylases. These factors and others were rigidly controlled in the routine experiments.

The conclusions drawn were as follows:

1. *Temperature Relations of the Amylases:*

a. Under comparable conditions, the optimal temperatures of the amylases are, human > terrapin > fish.

b. Fish amylase acts more efficaciously at 3.5° than do terrapin and human amylases. The order is fish > human > terrapin.

2. *Factors Influencing the Optimal Temperature:*

a. The enzyme-substrate ratio has an effect. The larger the proportionate amount of enzyme, the higher the optimal temperature in a given period. This effect depends upon heat inactivation of the enzyme.

b. The longer the period of digestion, the lower is the optimal temperature. This also depends upon the heat inactivation of the enzyme.

I wish to thank the Zoölogy Department of Duke University, and especially Professor F. G. Hall, for the facilities granted me while carrying on this work. A portion of the experiments were done at the United States Bureau of Fisheries Laboratory at Beaufort, N. C. I acknowledge my indebtedness to the Director of the station, Dr. H. F. Prytherch.

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THE STRUCTURE AND REACTIONS OF THE TENTACLES OF TEREBELLA MAGNIFICA W.

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Terebella magnifica Webster is a typical sedentary tube worm found under stones in the shallow water of the Bermuda Islands. Like many related forms it possesses numerous tentacular filaments which function in the building of the tube, obtaining food, respiration, and protection. The remarkable feature of these tentacles is their ability to survive and react after removal from the body of the worm. This automaticity and their length, which often exceeds 50 cm. when extended, provides material for numerous physiological experiments. The present paper deals with certain structural aspects of these tentacular filaments and their reactions to light and electrical stimulation.

Specimens of *Terebella* were obtained in large numbers along the shores of Long Bird Island and were easily kept in shallow dishes in running sea water. When gravel was provided they built new tubes and reacted very much as in normal surroundings, except that when exposed to light the tentacles were always more or less contracted, while in nature worms were occasionally seen with their fully extended tentacles exposed to brilliant illumination. The tentacles, which number from 60 to 100 in a large specimen, may extend to a length several times that of the worm or may be tightly coiled in a mass only a few centimeters in length. Tentacles which had been removed from worms survived for twelve days in bowls of sea water provided the water was changed frequently. New tentacles grew at the rate of 1 to 1.5 cm. per day.

Figure 1 is of a section through the midregion of a tentacle. The greatest diameter is about 1 mm. Along one side extends a deep groove. This is lined with ciliated epithelium and the very active cilia beat constantly toward the base of the tentacle. Microscopic examination of a living tentacle shows a constant stream of small particles consisting of diatoms and other small organisms being swept along this groove toward the basal end. The edges of the groove may be tightly applied one to the other, forming a ciliated tube, or they may be applied by a sucking action to rocks and algæ where the food is swept into the tube.

Covering the exposed surface of the tentacle is an epidermis containing numerous mucous glands (Fig. 3), which are more highly specialized than in the earthworm. Upon stimulation with diluted acetic acid, these glands discharge with considerable force, expelling a stream of mucus which forms a filament several times the length of the cell. The discharged gland resembles very closely a discharged nematocyst.

Longitudinal muscle fibers are most numerous, but in addition there is a complicated system of circular, cross, and oblique fibers. This system of muscles enables the tentacle to execute its many movements.

The central portion of the tentacle is a space, possibly continuous with the cœlomic cavity of the worm, containing a fluid in which amœboid cells are numerous.

The nervous system of the tentacle consists of five longitudinal groups of fibers (Fig. 2) with regularly arranged branches. Two large bundles are located one on each side of the tentacle. A medium-sized bundle is found opposite the ciliated groove and between this and the large lateral groups are two smaller bundles. Fibers enter and leave these bundles at regular intervals by way of circular connections which may be seen on one side of the tentacle in Fig. 2 and in a longitudinal section of a tentacle in Fig. 4. These branches may be traced into the region of the ciliated groove, gradually decreasing in diameter as the distance from the longitudinal bundles increases. Because the only material available for histological study was fixed in Bouin's fluid, it was

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Figures 1–6 are photomicrographs taken at various magnifications.

FIG. 1. Cross-section of a tentacle near midregion.

FIG. 2. Detail of region of tentacle containing the longitudinal bundles of nerve fibers and on one side a circular nerve connective which joins these bundles and extends to the side with ciliated groove.

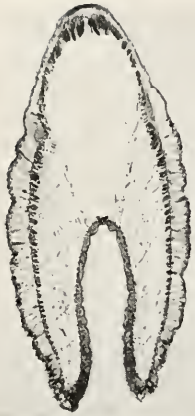
FIG. 3. The mucous glands of the epidermis.

FIG. 4. A longitudinal section through one of the large lateral nerve bundles showing the segmental arrangement of the circular connectives. (This photograph has been retouched in order to accentuate the outlines of the nerve tracts.)

FIG. 5. Details of the region of the lateral longitudinal nerve fiber tracts showing the characteristic thinning of the epidermis over these bundles.

FIG. 6. Some of the nuclei occasionally seen associated with the nerve fiber bundles. These may be nuclei of sensory cells.

CC = circular connective.
LLN = large longitudinal nerve fiber tract.
MN = median nerve fiber tract.
NU = nuclei of nerve or sensory cells.
SLN = small longitudinal nerve fiber tract.



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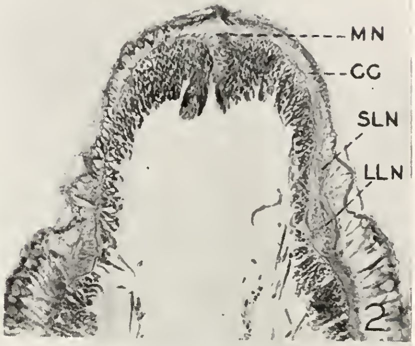

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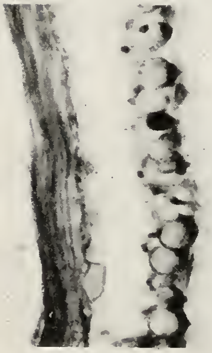

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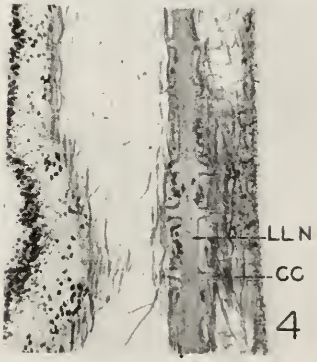

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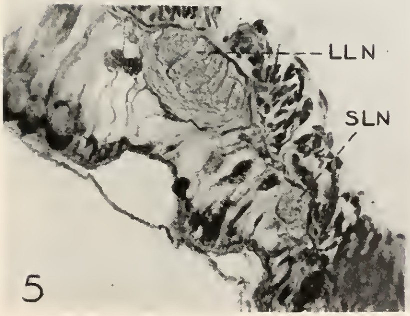

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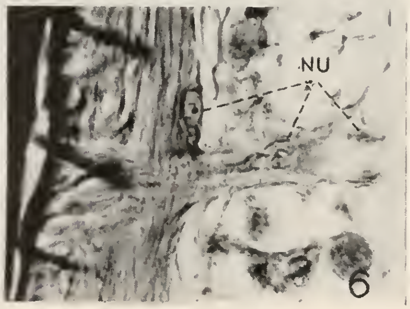

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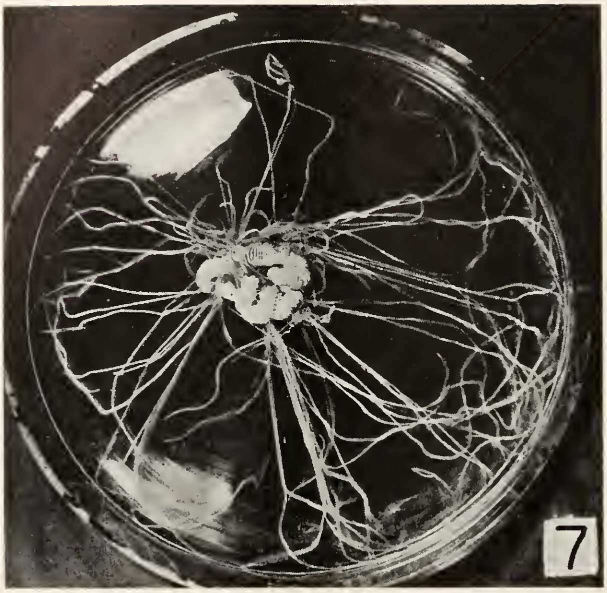

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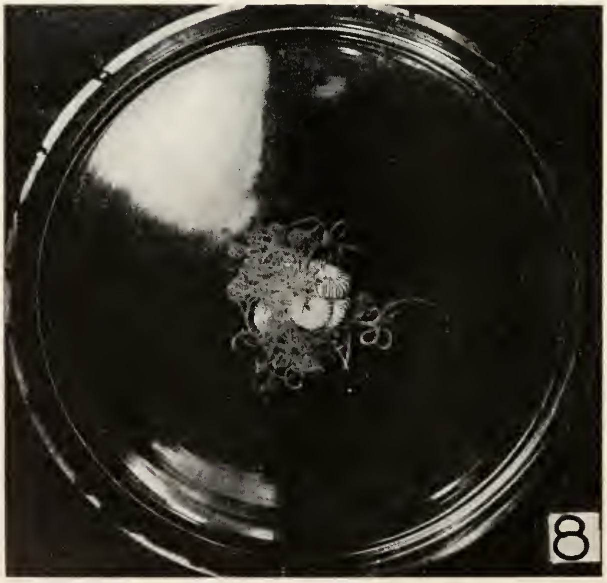

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Fig. 7. Photograph of dark-adapted worm taken with a brief flash of light. Note well-extended tentacles.

Fig. 8. Photograph of same worm as seen in Fig. 7 taken few seconds later. Note contracted tentacles.

impossible to trace individual nerve fibers and to locate readily sensory cells. The characteristic thinning of the epidermis over the lateral longitudinal bundles of fibers, seen best in Fig. 5, suggests that the photoreceptors may be located in this region. Nuclei of nerve cells are found in this vicinity and are usually more evident at points where circular connectives join the bundles (Fig. 6). The infrequent occurrence of nuclei within the bundles of nerve fibers leads to the conclusion that few intermediate neurones occur within the tentacle. A study of the responses of isolated and intact tentacles enables one to draw more definite conclusions regarding the functional arrangement of the nervous system of the tentacle.

Reactions of Tentacles to Light

When a specimen of *Terebella* has been in the dark for one-half hour or longer the tentacles are relaxed and extended to full length (Fig. 7). In this condition they are very sensitive to light and illumination with light of a few foot candles in intensity results in a rapid contraction of the tentacles followed by active writhing movements (Fig. 8). The reaction time, even at low intensities of illumination, was found to be too brief to measure accurately with a stop watch but was estimated to average about 0.5 second.

If a tentacle is removed and placed in sea water, in the dark, it behaves as though it were still a part of the animal. It becomes extended and quiescent after a period of dark adaptation and when illuminated reacts in as short a time as does an attached tentacle. The reaction time depends only very slightly on the intensity of illumination and over a range from 5 to 200 foot candles the change could not be measured accurately. It is probable that the use of brief flashes of light as employed by Hecht (1919–1920) in studying the sensitivity of the siphons of *Mya* would have yielded data which might have been studied quantitatively, as the reaction time is doubtless dependent on the length of the exposure period.

When a single attached tentacle is explored with a point source of light, beginning at the base and moving toward the tip, there is seldom a contraction until a portion of the distal third of the tentacle is illuminated. This might be accounted for by assuming a concentration of photoreceptors near the distal end or by assuming a nervous system which conducts primarily toward the base of the tentacle. Further evidence in support of one of these views will appear later.

One other interesting fact regarding the reaction to a point source of light is the independent behavior of single intact tentacles. The illumination of the tip of one tentacle and its subsequent withdrawal is rarely followed by movement of other tentacles. This indicates little coördination between the many tentacles.

Reaction of Tentacles to Electrical Stimulation

In order to obtain further evidence on conduction within the tentacle, electrical stimulation was used. An isolated tentacle was arranged in such a manner that on either side of the electrodes a portion of the tentacle passed over blocks of paraffin. The remainder of the tentacle was in contact with a glass plate where it was kept moistened with sea water. By means of an induction coil both single shocks and repeated stimuli were applied to a midregion of the tentacle. The lowest stimulus which elicited a response of the proximal half of the tentacle never caused a contraction of the distal half. The distal portion contracted only after repeated stimuli of high intensity.

Discussion

The reactions of isolated tentacles of *Terebella* to light and electrical stimulation indicate that there are probably direct connections between receptors and muscles as demonstrated histologically by Dawson (1920), in the ventral region of the earthworm. The experiments of Moore (1923) and Hess (1925), which confirmed Dawson's findings, also lend support to such a view.

The apparently greater sensitivity to light of the distal portion of a tentacle does not mean that the photoreceptors are most abundant in this region. Rand (1909) and Parker (1917) showed that in the tentacles of *Condylactis,* an actinian, conduction over nerves was always proximal. This supported the histological results on *Cerianthus* of Grošelj (1909), who found that almost all of the sensory cells of the tentacles were unipolar, with their fibrils extending without exception toward the base. Such a situation in the tentacles of *Terebella* would account for the apparently greater sensitivity of the distal portion of the tentacle, as a stimulus applied near the tip would have a greater effect on the tentacle, as a whole, than one applied near the base.

That the direction taken by the majority of fibers from receptors is toward the base is evidenced by the response to electrical stimulation. The similarity, in this respect, to the situation in the tentacles of actinians is of considerable interest. The polarity of the tentacle as regards conduction indicates that a nerve plexus or net probably does not exist here. This is in agreement with the recent work of Coonfield (1932) on earthworms.

SUMMARY

1. The tentacular filaments of *Terebella magnifica* W. are remarkably independent in their behavior after removal from the worm. Their functions are varied and they possess many features typical of an intact organism.

2. A deep ciliated groove acts as a collector and conductor of food to the body of the worm. The epidermis contains numerous specialized mucous cells.

3. The nervous system of the tentacle consists of five longitudinal bundles of nerve fibers with circular connectives or branches occurring at regular intervals.

4. Both intact and isolated tentacles are sensitive to light and in either case the reaction time over a fairly wide range of intensities is of the order of 0.5 second.

5. Local electrical stimulation elicits a response of only that portion of the tentacle proximal to the electrodes except when stimulating with rapid shocks of high intensity.

6. The experimental evidence indicates that the nervous system consists of direct receptor-effector elements which conduct primarily toward the base of the tentacle. There was no evidence which would seem to indicate the presence of a nerve plexus or net.

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THE CONCENTRATION OF EOSIN AND THE PHOTO-DYNAMIC EFFECT ON TENTACLES OF A TEREBELLID WORM

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An attempt to modify the sensitivity to light of the tentacles of *Terebella magnifica* Webster by means of various dyes occasioned the use of eosin, and necessitated an investigation of its toxicity in the presence of light. The results appear to be of sufficient interest to warrant their separate consideration.

The photodynamic action of light has occupied the attention of numerous investigators since it was first reported by Raab in 1900. Reviews of the literature by von Tappeiner (1909), Clark (1922), and Blum (1932) obviate the necessity of considering the subject historically. The question of immediate interest, that of the effect of the concentration of the dye, has been considered by Fr. von Tappeiner (1908), Pereira (1925), Dognon (1928), and others, but the results in experiments on living systems are for the most part incomplete or qualitative.

Materials and Methods

The tentacular filaments of *Terebella magnifica* W. provide excellent material for such studies. Their general structure and behavior to light has been discussed in an accompanying paper (Welsh, 1934). After removal from the worm the tentacles survive for several days in sea water and when illuminated continue to move about, coiling and uncoiling, as when intact. This constant activity is of considerable importance as it stops rather suddenly at the time of death and provides a definite end point for judging the time of killing. The relative transparency of the tentacles also permits the penetration of a large part of the incident light.

Several fluorescent dyes were employed in the investigation but the results from the use of only tetrabromfluorescein or eosin Y will be considered in this account. The eosin used was a product of the National Aniline and Chemical Co., Shultz No. 587, having a total dye content of 89 per cent. This was made up in sea water to give a one per cent stock solution by total weight. This stock solution was guarded against exposure to bright light. After a number of preliminary experiments the eosin was used in the following final dilutions; ½, 1, 5, 10, 15, 20, and 25 drops of stock solution to 25 cc. of sea water. It is unfortunate that the dilutions were not made in some other manner, as it is possible to calculate the normality only roughly; but the fact that care was used to control the drop size gave concentrations which for the purposes were sufficiently constant.

The several dilutions of eosin were placed in flat-bottomed glass dishes, 47 mm. in diameter, which gave a 15 mm. depth of solution. This thickness of the layer of eosin in sea water was a nearly constant factor, as the tentacles rested at the bottom and their movement exposed all surfaces to light of the same intensity. Sunlight was used as a source of illumination and the results reported were obtained from experiments performed on cloudless days, near midday, when the rays were essentially perpendicular. The tentacles were exposed to light immediately after being placed in the solutions.

TABLE I

Killing-time in minutes for four sets of tentacles at several concentrations of eosin. (Concentration = drops of 0.89 per cent solution to 25 cc. sea water.) Temp. 32° C. ± 1°. Experiments performed in sunlight, near midday.

Concentration eosin	Time for killing				Av.
	min.	*min.*	*min.*	*min.*	*min.*
Control	—	—	—	—	—
½	60	80	50	35	56.2
1	36	35	40	30	35.2
5	28	25	22	22	24.2
10	20	18	20	20	19.5
15	23	22	25	24	23.5
20	26	25	30	27	27.0
25	30	28	32	31	30.2

The small containers with the eosin and tentacles were placed in a white enamelled pan and surrounded with water in order to maintain a fairly constant temperature. This averaged about 32° C. Such a temperature was somewhat higher than that normally experienced by the worms but did not appear detrimental to the controls which were run in sea water with each experiment.

RESULTS

Upon exposure to light the tentacles in all of the several dilutions of eosin exhibited greater activity than the control in sea water. They would coil tightly and then rapidly uncoil. This continued until the epidermal cells began to plasmolyze, when the tentacles straightened, exhibited twitching movements, and soon ceased all activity. This cessation of movement was used as the endpoint and although it did not necessarily indicate the complete death and destruction of all the cells, it marked a point beyond which muscular movement did not occur. It was soon evident that this point was first reached at a concentration of ten drops of stock solution of eosin to 25 cc. of sea water. As the concentration of eosin increased or decreased in relation to this solution the killing-time increased.

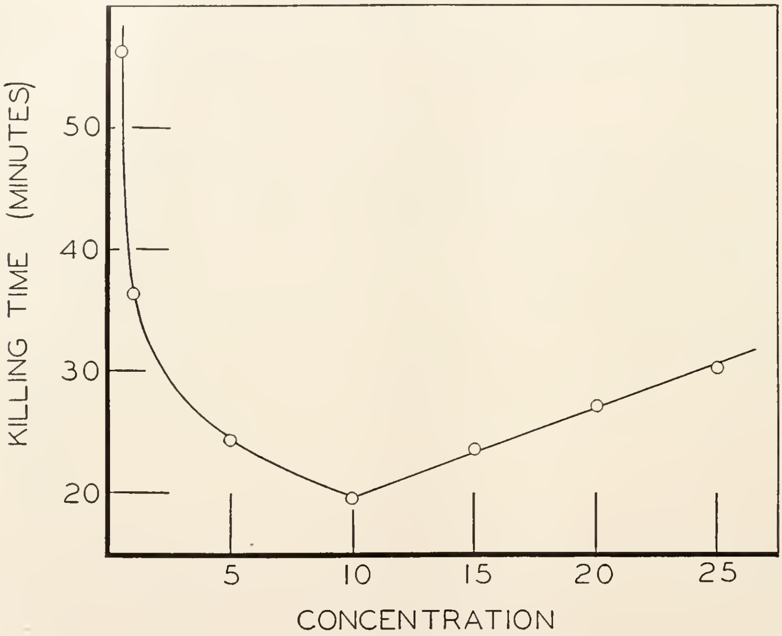

FIG. 1. The average times for killing, given in Table I, are shown plotted against the concentration of eosin. Concentration = number of drops of an 0.89 per cent solution of eosin in 25 cc. of sea water.

Table I gives the times for killing, at the several concentrations, for four separate experiments. In no instance was the control noticeably affected during the exposure although a period of illumination of several hours resulted in an earlier death of the tentacle than if the same were kept in diffuse light. It should also be mentioned that, in the diffuse light of the laboratory, tentacles would live for at least three days in the concentration of eosin which resulted in most rapid killing in direct sunlight. The averages of the times for killing are shown graphically in

Fig. 1. At the lowest concentration the average time for killing was 56 minutes. The times decreased regularly to the 10 drop concentration at which the variations were small and the average killing-time 19.5 minutes. A smooth curve may be drawn through the points obtained and the curve would approach more closely the abscissa were it not for a secondary process entering in which causes an increase in the killing-time. The secondary process is unquestionably due to a shielding effect resulting from an absorption of light by the overlying layer of eosin. It probably follows Beer's Law for the absorption of light. Exceptions to this law are known, but in general the absorption of light by a solution depends on the molecular concentration if the thickness of the absorbing layer is kept constant. This screening effect, which begins to reduce the photodynamic action of the eosin, results in an increase in killing-time and the averages now bear a linear relationship to the concentration over the range employed. If the thickness of the layer of eosin were varied, it is probable that the relationship between concentration and killing-time at the higher concentrations would vary considerably. However, another variable, that of the diffusion of oxygen, would enter in to complicate the matter, as the importance of oxygen in photodynamic processes has been repeatedly demonstrated.

The data of Fr. von Tappeiner (1908) obtained from a study of the effect of concentration of eosin on the hemolysis of red blood cells are in essential agreement with the above. A minimum time for hemolysis of 17 minutes was found at a concentration of 1/2000 N eosin. At concentrations above and below this the times increased. At a concentration of 1/40,000 N the time had increased to 44 minutes and at the highest concentration of 1/200 N the time for hemolysis was 34 minutes. The observations of Pereira (1925) on the combined toxic action of eosin and light on *Arbacia* eggs, sperm, and larvae were qualitative only, but indicated a definite effect of concentration of the dye. Dognon (1928) studied the effect of the concentration of several fluorescent dyes on the killing of paramecia and obtained a definite relationship between concentration and killing-time although he employed only concentrations below that which produced killing in a minimum time.

Results by Gros (1901) on the rate of bleaching of fluorescein in light indicate that at a given concentration the rate of bleaching is at a maximum and that increase or decrease in concentration produces a decrease in rate of bleaching. Blum (1932) mentions other such experiments on non-living systems, all of which yielded essentially similar results.

It would be of interest to employ screens of eosin varying in concentration, while keeping tentacles in a given concentration, in order to separate the absorption effect from the photodynamic action. Results obtained in this manner should yield to more precise analysis and permit the formulation of significant mathematical equations to explain the combined effect.

It is evident from the results obtained that the concentration of the fluorescent material is a limiting factor in determining the rate of a photodynamic process. When all other factors are constant the photodynamic effect increases with the concentration of the dye up to a certain point, beyond which there is a decrease due to the absorption of light by the layer of dye. Over the range of concentrations employed in the present study the times for killing, above the minimum, are apparently directly proportional to the concentration of the sensitizing material. Over a wider range of concentrations this relationship probably does not hold, as appears to be indicated by other similar investigations.

SUMMARY

The tentacles of *Terebella magnifica*, W. are satisfactory for the study of certain photodynamic phenomena. The toxic effect of eosin in the presence of light depends upon the concentration of the dye. As the concentration increases, the killing-time decreases to a minimum and then increases linearly over a certain range. This secondary increase is probably due to absorption of light by the overlying layer of eosin.

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THE MOBILIZATION OF CALCIUM DURING DEVELOPMENT

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I

According to Plimmer and Lowndes (1924) (Needham, 1931), the shell membrane of the hen's egg contains only a negligible amount of calcium before the seventh day of incubation. After that the quantity increases sharply and reaches its high point in the neighborhood of 8 mg. CaO on the twenty-first day. In what form is this calcium present?

It was suggested by Tangl (1908) that the embryo eliminating CO_2 itself created conditions progressively more and more favorable to the transformation of the insoluble $CaCO_3$ of the shell into the soluble bicarbonate. A working model of this idea was made by Buckner, Martin, and Peter (1925), who passed a stream of carbonated water through an empty eggshell and demonstrated not only the presence of $CaHCO_3$, but the free diffusion of the bicarbonate from the shell through the adhering shell-membranes. It was inferred "that during the first 9 days of incubation, before the allantois had touched the shell membrane, a water solution containing carbon dioxid given off by the embryo diffuses through the white and membranes to the shell, there forming calcium bicarbonate which diffuses back to the embryo, where it is metabolized. After the ninth day, when the allantois touches the shell membranes, it is reasonable to believe that the shell then gives up calcium bicarbonate to the blood stream as it discharges carbon dioxid."

Is calcium bicarbonate demonstrable in the shell membrane? When the shell membranes were removed from six unincubated Rhode Island Red eggs, and boiled in CO_2-free distilled water, the vapors produced no visible precipitate in $Ba(OH)_2$. Such membranes are not calcium-free, but if they harbor any as bicarbonate its concentration is too low to be detected in this way. However, when this experiment was repeated with the same number of membranes from groups of eggs incubated respectively for 7, 14, and 19 days, precipitates of $BaCO_3$ were formed, and, as might be expected, were progressively more voluminous. For our purposes this qualitative test is sufficient as it harmonizes completely with the implications of Plimmer and Lowndes (*loc. cit.*) and of Buckner, Martin, and Peter (*loc. cit.*).

II

Since $CaHCO_3$ is present in the shell membranes after one week of incubation, Ca must leave the shell.

The first convincing proof that the shell actually loses Ca, was secured on Plymouth Rock hens by Tangl (*loc. cit.*) and Hammerschlag. These investigators found the following average differences between eight unincubated eggshells and fifteen shells left after hatching. (See Table I.)

TABLE I

*Plymouth Rock Eggshells (with Shell Membranes)**

	Before incubation		After hatching		Loss
	per cent	*grams*	*per cent*	*grams*	*grams*
Dry weight		5.76		5.32	0.440
Organic substance	46.43	2.674	46.06	2.450	0.224
Calcium	37.19	2.142	37.14	1.976	0.166
All else	16.38		16.80		

* Since the analyses of the incubated shells include some Ca removed from the shell but retained by the shell membranes, Tangl's value of 166 mg. for the Ca-loss of the shell itself is probably too low. On the basis of Plimmer and Lowndes' figures (*loc. cit.*) a correction of 4.5 mg. seems justified.

Tangl (*loc. cit.*) comments on the constant relation between organic substance and calcium and suggests the possibility that the embryo derives calcium from organic and inorganic sources in the proportion in which such compounds are present. Referring embryonic Ca accession to $CaCO_3$ lost by the shell accounts for only half the calcium gained during development by the chick and its foetal membranes.

Neither Tangl nor Needham (*loc. cit.*) comment on an inconsistency in these data. If, as the analyses show, the Ca-organic-substance ratio remains constant throughout development, one would expect the same constancy in that part of the shell system which is dissolved. However, the 440 mg. of shell that have disappeared seem to have contained 37.73 per cent of Ca, and 50.45 per cent of organic substance. Is this an accumulation of experimental errors or is the absorption of organic substance and calcium differential?

According to these analyses, total shell weight decreases during incubation, but the concentration of calcium remains almost constant.

III

To verify this percentage constancy on individual eggs requires methods somewhat different from those previously employed. The eggs we used were all laid during March and April by one Rhode Island Red hen, controlled as to diet and kept monogamous. These eggs were incubated at 39.5° C. in an atmosphere sufficiently moist to insure the highest rate of Ca-metabolism (Romanoff, 1929). After 7 days in the incubator a bit of shell was removed with some assurance that this particular egg would continue normal development at least up to the nineteenth day (Romanoff, 1931). Inasmuch as very little Ca is absorbed by the embryo during the first week of incubation our twelve-day period includes all except one or possibly one and one-half of the significant days. On the assumption that spiral descent in the oviduct assures a uniform concentration—not amount—of calcium in various parts of the shell, one should be able to secure reliable values for the percentage of Ca present in a given eggshell on the seventh day of incubation and again on the nineteenth day. It is desirable not to injure the shell membranes and essential to control evaporation. This was done by covering the exposed shell membranes with a layer of lens paper kept continuously moist and held in place by a bit of paraffin. Six individual eggs were studied successfully in this way. The Ca was determined as CaO by adapting the method given in Blasdale (1928). The results, including reductions to Ca, are given in Table II.

TABLE II

Concentration of Ca in Rhode Island Red eggs. Shells without membranes.

Egg	After 7 days incubation				After 19 days incubation			
	Dry wt. sample	CaO	Ca	Ca	Dry wt. sample	CaO	Ca	Ca
	mg.	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*per cent*
1	78.9	43.3	24.0	30.4	312.6	169.7	94.3	30.2
2	65.2	35.4	19.7	30.2	510.6	272.6	151.5	29.7
3	120.6	64.0	35.6	29.5	826.7	438.6	243.7	29.5
4	144.5	79.4	44.1	30.5	851.3	464.5	258.1	30.3
5	184.1	100.1	55.6	30.2	1516.0	814.5	452.6	29.9
6	188.2	105.0	58.4	31.0	1284.0	691.5	384.2	29.9
				Av. 30.6				Av. 29.9

Thus individual eggs when compared on the seventh and nineteenth days of incubation also yield essentially constant percentages of calcium.

Since the eggshells lose weight during incubation, this substantial constancy in the concentration of calcium implies an actual removal of Ca from the shell. The quantity mobilized can be computed from the preceding and other data.

The unincubated Rhode Island Red shell has an average dry weight in the neighborhood of 4.36 grams (cp. Needham). Assuming a common rate of Ca-metabolism in all hen's eggs that hatch under standard conditions in 21 days (Byerly, 1932), our shells at the end of the incubation period should have weighed close to 4.03 grams. On the seventh day of incubation the Ca indicated by our analyses is equivalent to 1.334 grams per shell; on the nineteenth day, to 1.205 grams. The loss of calcium from the shell itself is therefore of the order of 129 mg. Applying a conservative 2 per cent correction for the missing days in our experiment (cp. Needham, *loc. cit.*) and correcting Tangl's value for Ca retained in the shell membrane, we arrive at the following orders of magnitude:

Ca lost by Rhode Island Red eggshell	131 mg.
Ca lost by Plymouth Rock eggshell	170 mg.

IV

If the suggestion of Buckner, Martin and Pepper (*loc. cit.*) is correct and allantoic contact with the shell membranes actually enables the shell to give " up calcium bicarbonate to the blood as it discharges carbon dioxid," we should find definite evidence of such erosion.

In order to secure ocular proof, eggs were incubated for 20 days in fixed position. In this way, by keeping each blood vessel constantly at work on the same portion of the shell, we hoped to produce visible erosion grooves. To identify these, we illuminated the eggs before opening and traced the pathways of the major vessels with pencil on the outside.

Although the inside of a shell incubated for 20 days is distinctly rougher than the same surface in a comparable newly-laid egg, convincing identifications of the pathways traced on the outside and the suspected erosion grooves within could not be made by this method alone. We therefore resorted to staining. Other things being equal, a penetrating stain should enter most rapidly where the shell is thinnest. After several failures we tried an alcoholic solution of methyl red. The shells were dipped into the solution, care being taken that no dye could reach the inner surfaces directly. After a considerable amount had penetrated through the shells, these were dried, and compared with unincubated shells treated in the same way. In this manner one can uncover, at times, in unincubated shells certain irregularities in thickness; however, the irregularities in the incubated shells form a branching and anastomosing system and the major ones corresponded to the light pencil-tracings made on the outside before the eggs had been opened. These differences are illustrated in Figs. 1 and 2.

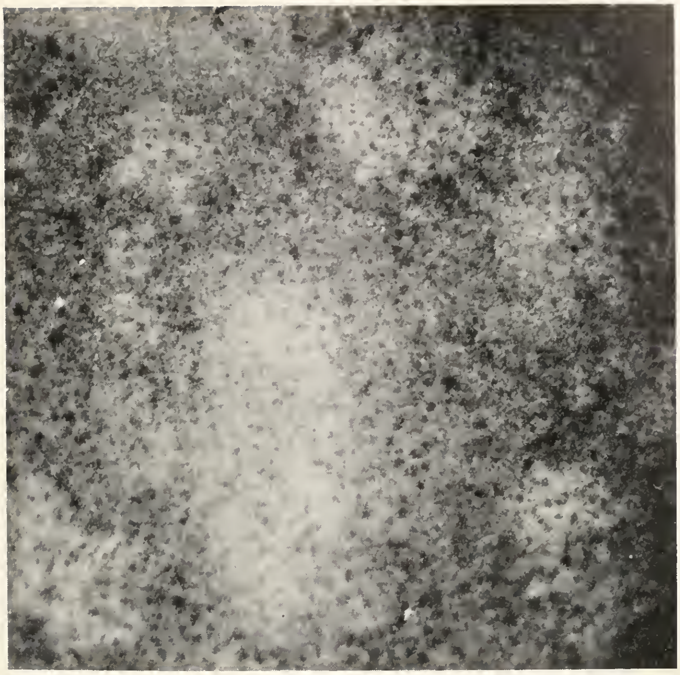

FIG. 1. Inside surface of unincubated eggshell. Treated with methyl red. Magnification about 5 ×.

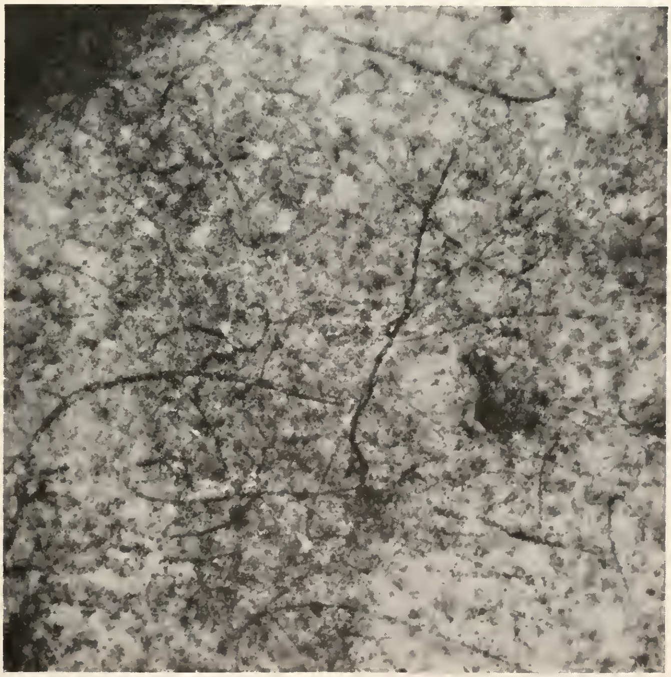

FIG. 2. Inside surface of incubated eggshell. Treated with methyl red to expose the erosion grooves left by allantoic blood-vessels. Magnification about 5 ×.

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NEMA PARASITES IN EMBRYO BATS[1]

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In examining a series of embryos of one of the phyllostomid bats, it was discovered that many of them were parasitized by nematode larvæ. Since infection at such an early stage seems to be almost or entirely unknown, a brief description of the circumstances may be of interest.

The bats in question are *Glossophaga soricina soricina* Pallas, of the South American family Phyllostomidæ; they were collected in November and December, 1933, at Arapuá in eastern Matto Grosso. The material available for study ranges from tubal ova to 15 mm. embryos. The early development is somewhat like that of the European bats. The ovum burrows completely into the uterine mucosa, so that the thick trophoblast is completely shut off from the uterine cavity by a decidua capsularis. The large yolk sac is formed early, before the amnion cavity and embryonic shield are established by the vacuolization of the ectodermic knob. No nemas have been observed in the uteri with the earliest stages. After the blastocyst has begun to expand, however, most of the ova show from one to three parasites located in either the yolk sac or the amniotic cavity. Table I gives the data.

Although all of the parasites found are larvæ, the ones in the older embryos are definitely more advanced in size and organization than those in the younger blastocysts. Most of them are spirally coiled, which makes it difficult to get an accurate measure of their size; fairly accurate measurements of one in embryo No. 73 gave 1.5 mm. as the length. They are not attached to embryo or membranes, but lie free in the extra-embryonic cavities. It is possible, of course, that they were attached and were dislodged by the fixing fluid, but the embryos show no trace of any injuries referable to an attachment of the parasites. In one case discussed below (No. 67) the parasites showed granules in the intestine that looked like the remains of ingested red corpuscles.

There are two conceivable routes of infection by the worms. There is a possibility that the larvæ come from the uterine cavity, boring through decidua capsularis, trophoblast, and entoderm into the yolk sac. This does not seem likely, as no parasites have been seen in uteri or tubes, although they would be readily detected in the cavities of these organs. The probable source of the nemas is the maternal blood stream, from which they would make their way through the placental tissues into the cavity of the ovum. There is one observation that seems to give direct support to this idea. In embryo No. 67 the entoderm and trophoblast covering one of the placental vessels (maternal) have been ruptured, so that the yolk sac is half filled with blood, and among the extravasated corpuscles are two nemas, the apparent agents of the injury. In one or two other vesicles a few maternal corpuscles were seen scattered in the yolk sac, but in most cases the penetration of the larva does not seem to cause bleeding, the tissues closing without leaving any trace of the worm's passage. At the time when the parasites bore through into the blastocyst, the yolk sac cavity fills almost all the vesicle, consequently this is where the nemas are usually found (but see No. 69 in Table 1). Later, as the amnion cavity expands and the lumen of the yolk sac is reduced to a very narrow slit, the worms seem to migrate through yolk sac and amnion into the cavity of the latter.

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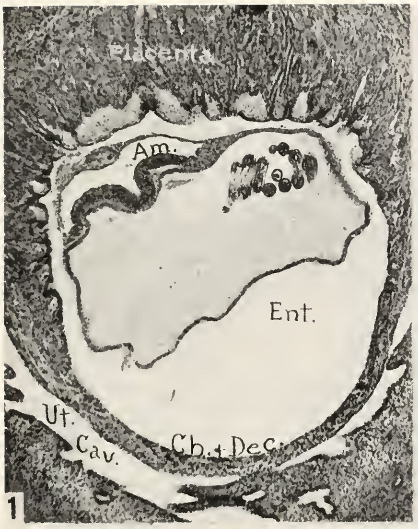
[1] The collection and study of this material were made under a Fellowship from the National Research Council, all the work except the actual collecting being done in the Anatomical Laboratory of the Harvard Medical School.

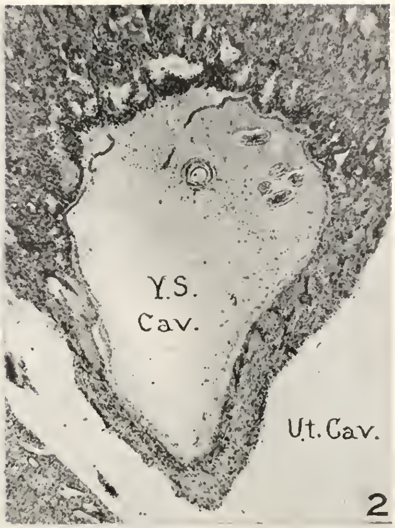
[2] National Research Fellow.

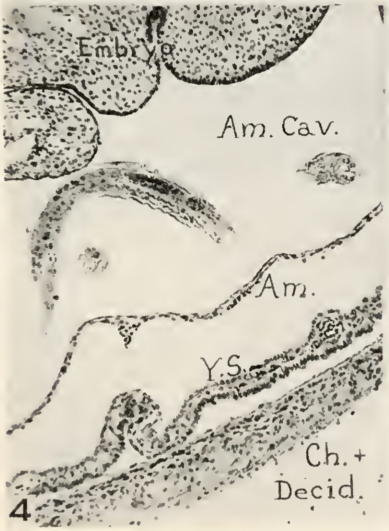
TABLE I

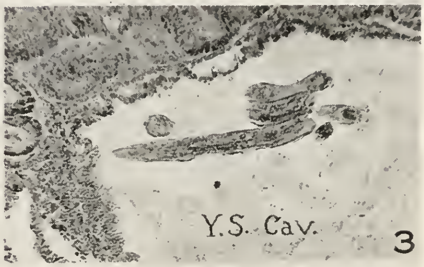
Embryo	Stage	No. of nemas	Location
1B 66	Solid ectodermal knob	0	—
1B 39	Amniotic cavity and embryonic shield	1	Yolk sac
1B 60	"	1	" "
1B 79	"	2	" "
1B 67	"	2	" "
1B 51	Primitive streak	2	" "
1B 82	" "	1	" "
1B 64	" "	0	—
1B 69	Neural folds	4	3 in yolk sac 1 between amnion and chorion
1B 80	" "	1	Yolk sac
1B 50	6 somites	0	—
1B 47	2.5 mm. embryo	1	Amniotic cavity
1B 38	2.7 mm. "	1	" "
1B 73	3.6 mm. "	1	" "
1B 42	4.0 mm. "	0	—
1B 54	6.5 mm. "	0	—
1B 85	10.0 mm. "	0	—

No evidence is available to show the ultimate fate of the parasites.









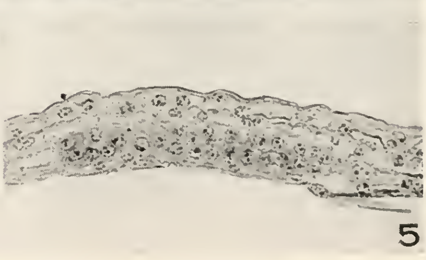


FIG. 1. Embryo 60. Section through amniotic cavity, embryonic shield, and yolk sac, showing a nema coiled up in the cavity of the yolk sac. The space between entoderm and chorion is due to shrinkage. × 60.

FIG. 2. Embryo 67. Section through yolk sac. Note the broken entoderm at the top, apparently caused by the two nemas, a few coils of which show in this section. The granular material surrounding them is blood, introduced by the migration of the parasites from the maternal blood vessels. × 60.

FIG. 3. Embryo 69. A nema in the yolk sac. × 120.

FIG. 4. Embryo 47. Three sections of a nema lying in the amniotic cavity. × 120.

FIG. 5. Part of a nema from embryo 38. Cuticle, ectoderm, and gut can be seen, as well as the elongated, darker-staining gonad. × 240.

Abbreviations: *am.*, *am. cav.*, amnion, amnion cavity; *ch.*, chorion; *decid.*, decidua capsularis; *ent.*, entoderm; *ut. cav.*, uterine cavity; *y. s.*, *y. s. cav.*, yolk sac, cavity of yolk sac.

In the oldest embryos (3.6 mm.) in which they have been found, the worms are still in the amniotic cavity. It is possible that they may persist here and so be lost with the discarding of the extra-embryonic membranes at birth. If so, their presence in the vesicles would have to be looked upon as being without significance. On the other hand, they may find their way into the embryo itself at some time before birth and so survive, in which case we would be dealing with a definite mechanism for the transmission of the parasite from mother to fetus. The high percentage of infection (65 per cent in the series examined) would seem to indicate that something more than mere accident is involved, but a study of a series of adults and of newly born individuals will be necessary before definite conclusions can be drawn.

It has not been possible to identify the larvæ up to this time. The slides have been examined by Dr. J. H. Sandground, of the Department of Tropical Medicine, who tells me that the worms are too immature to identify with certainty but that they may be the larvæ of some strongylid nematode. Nemas from the cœloms of some of the parent bats have been studied by Dr. Sandground and pronounced a new species of the filariid genus *Litomasoides;* they thus have no relationship to the parasites found in the embryos. I extend here my thanks to Dr. Sandground for his assistance in this phase of the investigation.

THE INFLUENCE OF HYPERTONIC AND HYPOTONIC SEA WATER ON THE ARTIFICIAL ACTIVATION OF STARFISH EGGS

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In the previous paper (2) the general conditions of artificial activation in the starfish egg were summarized briefly as follows: (*a*) a non-toxic penetrating acid (*e.g.* a fatty acid) acting alone, either in the presence or absence of oxygen, activates the egg completely and at a rapid rate which is closely proportional to the cH of the acid solution[1]; (*b*) hypertonic sea water may also cause complete activation but at a much slower rate and only in the presence of oxygen.

The important fact is that either of these two quite different changes of condition may produce the same physiological effect; but apparently they do so by inducing or promoting within the egg biochemical reactions of dissimilar kinds.[2] The hypothesis was proposed that the acid acts by catalyzing the hydrolytic decomposition of some ester-like compound (possibly phosphagen) normally present in the egg, and that the reaction which directly determines activation consists in the chemical union of a product of this hydrolysis with some other compound also present or available in the egg. This second compound appears to be formed (or its quantity increased) under the influence of hypertonic sea water in the presence of oxygen, the presumption being that the abstraction of water promotes its dehydrolytic synthesis from certain precursors. The combination of the two compounds produces a third compound, the activating substance, whose accumulation renders the egg capable of automatic development.

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[1] Heat activation is regarded as a special case of acid activation.

[2] Certain other characteristic differences in the physiological effect of the two treatments are in harmony with this conclusion. Thus, if the eggs are exposed briefly to heat (32°–35°) or acid (e.g., .004m butyric acid in sea water) within a few minutes (up to ten) after removal from the starfish, they not only show no activation but remain permanently immature with germinal vesicle intact (4). Hypertonic sea water, on the contrary, activates the eggs in a typical manner when the exposures are begun during this period. The immediate effect produced by the hypertonic sea water is apparently independent of the breakdown of the germinal vesicle; while the acid is quite without activating effect until this change has occurred, after which the egg is readily activated by both heat and acid. Apparently the substance which reacts with the acid is either absent or unavailable until the germinal vesicle has broken down.

According to this hypothesis the critical change determining activation is the chemical union of the hydrolytic product, which we designate as B, with the synthetic product, called S, to form the activating substance, A. This reaction (activation reaction) would then be represented by the equation

$$S + B = A,$$

and its velocity by the equation

$$V = K\ C_s C_b,$$

where K is the velocity constant of the reaction, and C_s, C_b the concentrations of S and B respectively.

B is regarded as being freed by the activating acid at a rate proportional (through a certain range) to the cH; it combines as rapidly as it is formed with S; hence the proportionality between the cH and the rate of activation. In normal untreated eggs the concentration of S (*i.e.*, C_s) is assumed to be a constant, fixed by the inherited constitution of the egg. With C_s thus constant, the rate of activation should be proportional to the concentration of the activating solution of acid, as we find (3). If, however, we vary C_s, as by previously treating the eggs with hypertonic sea water, the rate of activation in a constant solution of acid should vary correspondingly; under such circumstances this rate, according to the formula, would be proportional to C_s.

Experiment shows that unfertilized eggs treated for a brief period (5–40 minutes) with hypertonic sea water and then exposed to acid show the usual course of acid activation, but at an increased rate (Table I). This characteristic effect may be described by saying that the eggs are *sensitized* to the activating influence of the acid.[3] According to our hypothesis the effect follows from the dehydrolytic synthesis of additional S (called also the sensitizing substance). In this synthesis a number of simple molecules are assumed to interact to form a molecule of the more complex compound S; in the successive steps of any such synthesis the molecules are to be conceived as combining successively in pairs; and the rate of the total synthesis will be determined by the rate of interaction of the pair of molecules which combine most slowly.[4]

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[3] The analogy with the sensitization of photoreceptors to light during the process of dark-adaptation, which follows a very similar time course (6, 7), is pointed out below.

[4] The relation of oxygen to hypertonic activation suggests that the combination of molecular oxygen with some other compound may be the controlling reaction.

The course of hypertonic sensitization would then be expected to follow the curve of a bimolecular reaction. In the following experiments the rate of activation by a constant solution of butyric acid, .004M in sea water, has been determined after exposing the eggs for varying periods to hypertonic sea water of constant composition.

Action of Hypertonic Sea Water Followed by Acid

A typical experiment is summarized in Table I. In all experiments of this kind the eggs were placed, shortly after the breakdown of the germinal vesicle, in hypertonic sea water of a constant composition (100 volumes sea water *plus* 20 volumes 2.5 M NaCl). After varying intervals, usually 10, 20, 30 and 40 minutes, portions were transferred to normal sea water, where they remained 10 or 15 minutes; they were then transferred to .004 M butyric acid solution in sea water. From this solution portions were returned to normal sea water at regular intervals (usually of 1 minute between 1 and 10 minutes). The approximate percentages of eggs developing to blastulæ were later determined. Controls of eggs exposed to hypertonic sea water without acid treatment, and to butyric acid without hypertonic treatment, were always kept.

Table I

July 7, 1932. Eggs from several animals were removed at 3:40 and placed in hypertonic sea water at 4:10. Temperature of solutions and sea water 22°. In the fertilized control practically all eggs developed normally.

In hypertonic sea water (22°)	Interval in normal sea water	Percentages of eggs forming blastulæ after exposure to .004 M butyric acid (22°) for the times indicated (min.)								
		(Control: hyp. sea water alone)	2	3	4	5	6	7	8	9
min.	*min.*									
A. 0*			0	pr. 0	15–20	40–50	70–80	80–90	70–80	50–60
B. 10	10	0	pr. 0	*ca.* 1	30–40	*ca.* 90	70–80	*ca.* 50	30–35	*ca.* 10
C. 20	12	0	<1	*ca.* 20	55–65	60–70	*ca.* 50	*ca.* 5	0	0
D. 30	12	pr. 0	*ca.* 1	25–30	70–75	50–60	30–40	3–5	0	0
E. 40	12	<1	5–10	40–50	65–70	*ca.* 50	15–20	*ca.* 5	0	0

* Control: acid alone.

In this experiment the series treated with butyric acid alone (*A*) shows the typical increase in the proportion of favorably developing eggs with increasing time of exposure up to a definite optimum at about 7 minutes, after which there is a decline. With the eggs treated previously with hypertonic sea water a similar time curve of activation is seen in each of the series *B* to *E*, but the effective exposures (as measured by the formation of blastulæ) are in all cases briefer, and the more so the longer the treatment with hypertonic sea water. The optima are respectively *A*, 7–8 minutes; *B*, 5 minutes; *C*, 4–5 minutes; *D*, *ca.* 4 minutes; *E*, *ca.* 4 minutes.

In ten experiments of this kind in June and early July a similar result was found. In two of these (June 10 and June 11, 1931) the temperature of the solutions was 18° and the optimal exposures to the acid alone were longer (*ca.* 20 minutes). The other eight experiments are summarized in Table II; these were carried out under closely similar conditions; the temperature of the hypertonic sea water and butyric acid solution was 20°–22°, and the optimal exposures to the acid alone varied between 7 and 9 minutes. The table gives the results of exposures to butyric acid after treatment with hypertonic sea water $(100 + 20)$ for periods varying from 10 to 40 minutes. The single experiments are in good agreement, and the averages show a regular decrease of activation-time with increase in the duration of the hypertonic treatment. Up to 40 minutes few or no eggs develop to blastulæ as a result of the hypertonic treatment alone; but an exposure of 60 minutes to hypertonic sea water alone usually activates a considerable portion of eggs, so that the sensitizing effect then becomes difficult to estimate. In the experiments of June 17 and June 18 eggs exposed to hypertonic sea water for 60 minutes formed a considerable proportion of blastulæ without further treatment (*ca.* 10 per cent), and 50 per cent or more blastulæ with additional exposures of 1 and 2 minutes to butyric acid.

The data show that the action of hypertonic sea water in shortening the effective exposures to butyric acid is most rapid during the earlier part of the immersion and falls off regularly as time advances. In Fig. 1 the average optimal exposures to acid (optimal activation-times) are plotted against the times in hypertonic sea water. The curve drawn is the theoretical bimolecular isotherm, the assumptions being (1) that the rate of the activation-process (the reciprocal of the activation-time) is proportional to the concentration of *S*, and (2) that the rate of formation of *S* is controlled by the interaction of two precursor molecules of equal concentration $(P + Q = S)$. The simple bimolecular formula is used,

$$\frac{1}{t} \cdot \frac{x}{a(a-x)} = K;$$

a represents the initial concentration of P or Q, and x the concentration of S formed by the time t. The initial concentration, a, is equal to the difference between the concentration of S when the reaction is complete,

TABLE II

Optimal exposures (minutes) to butyric acid solution (.004 M in sea water at 20–22°) and percentages of eggs forming blastulæ (in brackets) after previous treatment with hypertonic sea water for varying periods.

Experiment no.	Date	Durations of previous exposure (minutes) to hypertonic sea water (20°–22°)						
		0 (control)	10	15	20	30	40	60
1.	June 17	9 (75–80)			5 (30–35)		4 (30–40)	1–2 (*ca.* 50)
2.	June 18	7 (80–90)			5–6 (*ca.* 50)		4 (*ca.* 50)	1–2 (*ca.* 50)
3.	June 29	7 (*ca.* 50)	5 (35–40)		4 (20–30)	4 (20–30)		
4.	June 30	7–8 (65–75)	6 (50–60)		5 (40–50)	5 (35–45)	4 (35–45)	
5.	July 6	8 (70–80)	5 (55–65)		4 (30–35)	3 (35–40)	2–3 (35–45)	
6.	July 7	7 (80–90)	5 (80–90)		4–5 (55–70)	4 (70–75)	4 (65–70)	
7.	July 8	7 (75–85)		5 (50–60)		4 (*ca.* 50)	3 (*ca.* 40)	
8.	July 11	8 (70–80)	5 (60–70)		5 (*ca.* 50)	3–4 (30–35)	3 (30–35)	
Averages		7.5 min.	5.2 min.	5 min.	4.7 min.	3.9 min.	3.5 min.	

and its concentration at the beginning of the reaction (*i.e.*, before any hypertonic treatment); this difference is regarded as proportional to the difference between the optimal activation-times at these two periods. a-x is the concentration of S yet to be formed at any stage in the reaction; *i.e.*, when the latter is complete a-x becomes zero. S is steadily being formed during the immersion in hypertonic sea water but at a progressively decreasing rate. The time required for complete transformation of the precursors into S is estimated by the extrapolation of the curve to its asymptote or final level. This time is estimated at about 90 minutes, and the limiting value of the activation-time as 2 minutes. The calculated curve is drawn on this basis (see Table III), and the position of the points representing the observations shows a good correspondence with the theory.[5]

TABLE III

The final concentration of S is regarded as that corresponding to an optimal activation-time of 2 minutes. The value of a is then $7.5 - 2.0 = 5.5$. The additional concentrations of S formed after the times t are regarded as proportional to the decreases in the optimal activation-times (column 3).

Time in hypertonic sea water (t)	Optimal activation times	Additional S formed at time t (x)	P or Q at time t (a–x)	K	Activation-times calculated from average value of K
min.	*min.*				*min.*
0	7.5	0			
10	5.2	2.3	3.2	0.013	5.36
20	4.7	2.8	2.7	0.010	4.43–
30	3.9	3.6	1.9	0.0115	3.88
40	3.5	4.0	1.5	0.012	3.56
				(average 0.0115)	

It is interesting to note that the curve resembles in its general character the curves representing the course of sensitization to light during the process of dark-adaptation, as recently investigated by Hecht. He regards dark-adaptation as resulting from the accumulation of a light-sensitive compound formed by a bimolecular (*i.e.*, bimolecularly-controlled) reaction, and he has shown that many forms of dark-adaptation follow a curve of this type (7). Hartline has recently shown the same for the eye of *Limulus* (8). Hecht's curve of the light-sensitization of *Pholas* during 2½ hours in the dark (9) may be compared with Fig. 1.

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[5] The estimated activation-time of 2 minutes corresponding to the maximal concentration of S cannot be determined directly, for the reason that after sufficient exposure to hypertonic sea water many eggs develop completely without any acid treatment. This has already occurred at 60 minutes in the two experiments of June 17 and 18 (Table II). The optimal exposures to hypertonic sea water alone at 20° are between 75 minutes and 2 hours; this would indicate that maximal sensitization has then been reached. On the average there is not much difference in the effects of exposure between 75 and 120 minutes. Runnström finds that acid is formed in the sea-urchin egg after prolonged exposure to hypertonic sea water (6); such an effect would account for the formation of the hydrolysate B required for the assumed reaction $S + B = A$, and would also explain why after 60 minutes in hypertonic sea water the effect of the butyric acid solution is greater than would otherwise be expected.

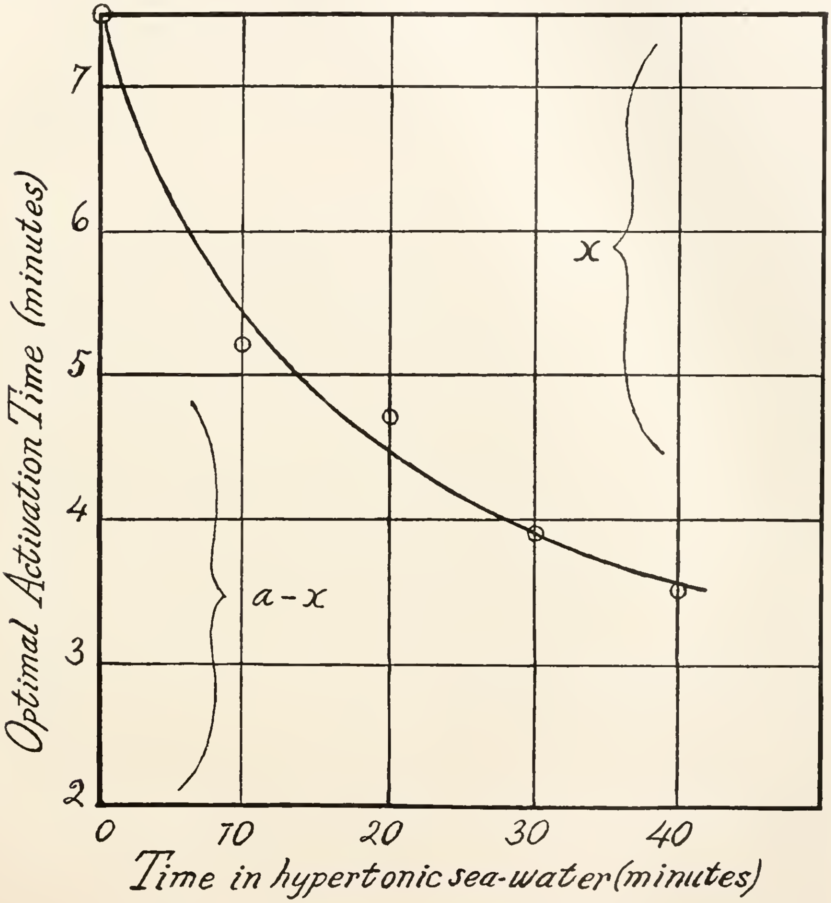

FIG. 1. Ordinates: average optimal activation-times (minutes of exposure to .004 M butyric acid in sea water). Abscissæ: previous exposures to hypertonic sea water (minutes). Temperature 20°–22° (see Table II).

A constant light-intensity (analogous to the constant acid-concentration used in the present experiments) was used for stimulation, and the reaction-time (= activation-time) was found to decrease progressively to a minimum, in a manner resembling closely that just described.

The fact that the sensitizing effect of hypertonic sea water appears so soon after immersion and that the curve shows so steep a descent during the first few minutes, indicates that the loss of water from the site of the sensitizing reaction is rapid and reaches equilibrium almost immediately. Localization of the reaction in the most superficial layer of the egg cortex would thus be indicated, possibly the layer immediately beneath the outer pellicle; the latter is apparently scleroprotein in nature, and is separated on activation as the fertilization membrane (10). This would imply that a more internal layer controls the osmotic loss of water from the egg as a whole, a process requiring apparently about 10 minutes to reach equilibrium, as indicated by the curve of shrinkage in sea water made hypertonic with glycol (11, 12).

Butyric Acid Followed by Hypertonic Sea Water

When a brief exposure to butyric acid, sufficient for partial but not for complete activation, precedes the hypertonic treatment, the results are less regular than with the reverse order. The effective exposures to hypertonic sea water are shortened, but the increase in rate of response to this agent (as measured by the decrease in effective exposures) shows no very definite relation to the time in butyric acid solution. When the exposures to acid are sufficient for complete or almost complete activation, the subsequent treatment with hypertonic sea water is ineffective or injurious; while with longer than optimal exposures it is definitely injurious.

These effects are illustrated by the two series summarized in Tables IV and V. The eggs were placed, shortly after the breakdown of the germinal vesicle (30–35 minutes after removal from the starfish), in .004 M butyric acid in sea water for the times indicated; they were then returned to normal sea water, and after 15 or 20 minutes they were exposed to hypertonic sea water. The percentages forming blastulæ and gastrulæ are given as before; the results of single treatments with acid or hypertonic sea water are also given. Both lots of eggs were normal and responded normally to sperm fertilization. Table IV gives the results observed with briefer exposures to acid, and Table V those with longer exposures ranging from sub-optimal (6 minutes) to supra-optimal (12 minutes).

In fourteen experiments of this kind during the summers of 1931–32 the results were in general similar but with considerable variation in detail. The previous treatment with acid appears to disturb the regularity of the response to hypertonic sea water, possibly because of changes in the osmotic properties of the surface layer; and regular quantitative relations of the kind observed with the reverse order of treatment are difficult to discern. The effective exposures to hypertonic sea water are, however, almost invariably shortened by brief treatment with butyric acid; this effect may be attributed to the formation of the acid-product, *B*.

Table IV

Experiment of June 26, 1931. Concentrations of hypertonic sea water and butyric acid solution the same as before; temperature of both *ca.* 20°.

Time in acid solution	Durations of exposure to hypertonic sea water (minutes) and percentages of resulting blastulæ								
	(Control: acid alone)	10	20	30	40	50	60	75	90
min.									
A. 0*....		0	0	<1	20–25	30–40	50–60	60–70	65–75
B. 1.....	0	*ca.* 1	20–25	30–40	40–45	*ca.* 50	40–50	*ca.* 50	
C. 2.....	0	*ca.* 1	*ca.* 10	40–50	*ca.* 50	50–60	*ca.* 50	30–40	
D. 4.....	0	*ca.* 1	*ca.* 1	10–15	40–50	*ca.* 50	50+	50–60	
E. 6.....	15–20	*ca.* 1	*ca.* 1	*ca.* 1	*ca.* 1	*ca.* 1	3–5	3–5	

* Control: hypertonic sea water alone.

Table V

Experiment of June 29, 1932. Similar conditions. Temperature 21–22°.

Time in acid solution	Exposures to hypertonic sea water (minutes) and percentages of blastulæ							
	(Control: acid alone)	15	30	45	60	75	90	105
min.								
A. 0*......		<1	2–3	*ca.* 50	*ca.* 90	85–90	75–85	60–70
B. 6......	60–65	60–70	70–80	60–70	65–75	60–65		
C. 8......	90+	50–60	50–60	30–40	30–40	25–35		
D. 10.....	30–40	*ca.* 5	*ca.* 1	<1	<1	<1		
E. 12.....	<1	<1	<1	0	0	0		

* Control: hypertonic sea water alone.

Hypotonic Sea Water Followed by Butyric Acid

Theoretically we should expect that if hypertonic sea water acts by reducing the concentration of water at the site of the activation reaction, thus shifting the equilibrium in the direction of the further dehydrolytic synthesis of the sensitizing compound, *S*, the action of dilute sea water would be in the opposite direction; *i.e.*, previous exposure to hypotonic sea water should lengthen the activation-time in butyric acid solution.

Experiments were performed similar to those already described, but with hypotonic substituted for hypertonic sea water. The following dilutions of sea water were used: 15, 20, 25, 30, 35 and 40 volumes distilled water in 100 volumes of the mixture (*i.e.*, 85, 80, 75 etc. volumes per cent sea water). The eggs were placed in the hypotonic medium for periods of 15 to 30 minutes (*ca.* 20°), returned to normal sea water for 10 or 15 minutes, and then exposed to .004 M butyric acid in normal sea water.

Eggs treated with dilute sea water and then returned to normal sea water without further treatment show no membrane-formation or other signs of activation. The osmotic intake of water has thus by itself no evident activating action. Previous treatment with slightly dilute sea water (80 to 85 volumes per cent) has in some cases been followed by retardation of acid-activation, but the results have not been uniform. With the higher dilutions (75–60 volumes per cent sea water) the reverse effect was usually seen. This apparent acceleration of acid-activation by the more dilute sea water appears to be a summation effect. The conditions suggest that in the more dilute solutions the hypotonic distension has in itself some activating (acid-producing) effect which may compensate or mask the influence of dilution on the *S*-producing reaction.

Influence of Hypertonic and Hypotonic Sea Water on Activation by Warm Sea Water (32°)

During the course of maturation starfish eggs lose their responsiveness to heat-activation sooner than to acid-activation. After the separation of the first polar body (60–65 minutes after removal in sea water at 20°), eggs exposed to sea water at 32° for the optimal time give few if any blastulæ, while the response to butyric acid remains normal for some time longer. After the second polar division (90–100 minutes after removal), the response to acid declines rapidly. At 2 hours few if any eggs form blastulæ.

The time during which experiments with combined heat and hypertonic treatments can be carried out with fully responsive eggs is thus somewhat abbreviated. Nevertheless, the general type of result is the same as with acid-activation. In eggs treated previously with hypertonic sea water the response to heat is definitely accelerated. This has been established in a large number of experiments, the details of which need not be given. The results have shown somewhat less quantitative regularity than in the case of acid-activation; this difference may be attributed partly to the greater difficulty of controlling the conditions and partly to variations in the acid-producing reaction within the egg.[6]

The following experiment (July 6, 1933) is typical (Table VI). Eggs were exposed to hypertonic sea water (beginning 30 minutes after removal) for (*B*) 15 minutes and (*D*) 38 minutes, returned to sea water (20°) for a short time, and then exposed to the warm sea water (32°). The controls (*A* and *C*) were treated (at the same interval after removal as *B* and *D*) with warm sea water alone.

With all times of exposure to 32° (from 2 minutes on) activation was more rapid in the eggs treated previously with hypertonic sea water. The eggs in Experiment *C* were exposed after the first polar division and show only slight response; in Experiment *D* maturation has been delayed during the stay in hypertonic sea water and responsiveness remains good at the same interval after removal. Hypertonic sea water has been found uniformly to retard the loss of response to heat-activation, besides decreasing the effective times of exposure.

Table VI

Treatment of eggs	Result
A. 32° alone in sea water, beginning 51 min. after removal	Optimum at 6 min.: 60–65% blastulæ
B. Hypertonic sea water for 15 min.; normal sea water 12 min.; then 32° in sea water, beginning 52 min. after removal	Optimum at 4–5 min.: 65–75% blastulæ
C. 32° alone, beginning 80 min. after removal	Optimum at *ca.* 5 min.; few blastulæ (*ca.* 5%)
D. Hypertonic sea water 38 min.; sea water 8 min.; 32° in sea water, beginning 81 min. after removal	Optimum at 4 min.; many blastulæ (*ca.* 90%)

Experiments with hypotonic sea water have so far been few, and the influence on activation times was not constant. In this case also the loss of responsiveness is delayed during immersion in the dilute medium.

Hypertonic Sea Water and Acid Acting Simultaneously

In the experiments so far described, where fatty acid and hypertonic sea water were used in combination, the two treatments were applied successively, with a brief interval in normal sea water. When the treatments are applied simultaneously, *i.e.*, when the eggs are exposed to hypertonic sea water containing .004 M butyric acid, a somewhat unexpected result is obtained. The activating action of the acid is markedly retarded, and the period during which this action is exhibited is correspondingly prolonged.

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[6] If we regard heat-activation as a special case of acid-activation, it would seem that some reactant or catalyst in the acid-producing reaction within the egg is lost or destroyed near the time of the first polar division. The other substances required for activation by acid (whether the acid is formed within the egg or enters from without) appear to remain unaltered for some time longer, at least until the second polar division, after which the response to all types of activation, including sperm-fertilization, rapidly declines.

The following experiment will illustrate (June 22, 1933). Eggs of the same lot were treated simultaneously during the prematurational period with .004 M butyric acid dissolved (*A*) in normal sea water and (*B*) in hypertonic sea water, and then returned to sea water. Table VII gives the durations of exposure and the approximate percentages of eggs forming blastulæ.

Eight experiments of this kind were carried out during June 1933, and in all a similar effect was shown, with some variation in degree. The delay in activation appears to be correlated with a delay in the structural change associated with activation. In all experiments membrane-formation was prevented or retarded, the range of effective exposures was lengthened, and the breakdown following supra-optimal exposures was delayed. In one case 50 per cent of eggs formed blastulæ after an exposure of 24 minutes.

TABLE VII

Times of exposure (minutes at 20.5°) and percentages of blastulæ

Solution used	2	4	6	8	10	12	14	16	18	20	22	24
A. .004 m. butyric acid in normal sea water...........	*ca.* 1	*ca.* 5	50	80–90	*ca.* 80	20–25	<5	0				
B. .004 m. butyric acid in hypertonic sea water.......	*ca.* 1	*ca.* 1	<5	15–20	30–40	70–80	70–80	60–70	*ca.* 60	20	10	0

There is much evidence that a structural change in the surface-layer or "cortical zone" of the egg is a constant factor in both normal and artificial activation in many species; this evidence is seen in membrane-formation in echinid eggs, increase of permeability to water and dissolved substances, visible breakdown of the cortical layer in annelid eggs, and similar effects. Conversely, agents which alter the structure of the surface-layer, such as cytolytic compounds, mechanical agitation, and strong electric currents, have an activating effect.[7] The retarding influence of hypertonic sea water on acid-activation, shown in the experiments just described, may be interpreted as an expression of increased structural coherence or density resulting from the partial dehydration of the surface-layer. To the delay in the structural change corresponds the delay in the primary chemical reaction of activation. In the terms of our general hypothesis, the rate of union of the reactants S and B may be regarded as determined in part by the structural conditions (*e.g.*, permeability conditions) controlling the rates of diffusion at the site of the activation-reaction, as well as by the conditions of concentration already considered. Such a view would account both for the retardation of the reaction and for its being spread out over a longer period of time.

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[7] The fact that the progress of the activation-reaction in warm sea water has such a high temperature-coefficient (1), and that the reaction may be immediately arrested at any stage by returning the eggs to sea water, suggests again a dependence of activation on some readily reversible structural change. Activation

Nature of the Chemical Reactions of Activation

The special nature of the chemical reactions underlying activation is still obscure. In the former paper (2) it was pointed out that certain conditions of acid-activation (such as independence of oxygen-consumption) indicate a hydrolysis or other type of non-oxidative splitting process as the most probable type of primary reaction, and from the analogy with other cells (muscle, yeast, nerve) the splitting of a phosphagen ester was suggested. An attempt to test this hypothesis was made in the summer of 1932, by the analytical estimation of the free phosphate in the eggs before and after fertilization; but the analyses [8] (somewhat few in number) gave no indication of any change. A further corollary of the phosphagen hypothesis is that the other possible hydrolytic products might be concerned in the reaction-sequence of activation; but experiments in which eggs were warmed at 32° (1 to 9 minutes) in sea water containing the phosphagen-forming bases, creatin, arginin, and guanidin (0.1 to 0.4 per cent creatin hydrate, and hydrochlorates of the other bases in similar concentrations) also gave essentially negative results. If such compounds penetrate the egg in the warm sea water, as it seems reasonable to suppose—since (*e.g.*) hydrochloric acid in low concentration gives evidence of penetrating under these conditions (2)—they are without influence in the rate of the activation-reaction. Up to the present, at least, the phosphagen hypothesis has received no support from the experiments which it has suggested.

A similar uncertainty exists with regard to the nature of the acid

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arrested in this way can be renewed and carried to completion by a second exposure to heat, or by exposure to acid or hypertonic sea water (1).

[8] Carried out by Dr. Pei-Sung Tang, to whom my best thanks are due. Runnström also has been unable to find any change in the free phosphate of sea-urchin eggs after fertilization (6).

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which we have inferred—from the close resemblance between heat and acid-activation—to be formed within the egg during the exposure to warm sea water. If heat-activation is a form of acid-activation, the most obvious supposition would be that lactic acid is formed (within the egg-cortex) by glycolysis, very much as in the heat rigor of muscle. Increase in lactic acid following fertilization has recently been observed in sea-urchin eggs by Perlzweig and Barron (13). I have found, however, that iodoacetic acid, which blocks glycolysis in a large variety of cells, does not interfere appreciably with heat-activation in starfish eggs. In three successive experiments in which eggs (from three different lots) were warmed to 32° in sea water containing 0.02 per cent Na-iodoacetate the course of heat-activation was entirely normal, 60 per cent or more of the eggs forming blastulæ with exposures of 4 to 6 minutes. The only noticeable difference from the control (32° in normal sea water) was a slight shortening of the activation-time in the presence of iodoacetate and the production of a somewhat larger proportion of blastulæ. This result seems quite inconsistent with the glycolysis hypothesis of heat-activation, unless we assume absence of penetration, or that iodoacetate is without influence on the acid-forming reaction in these eggs.[9] The possibility may be considered that some acid other than lactic, *e.g.*, pyruvic, is responsible for heat-activation. Further experimental and chemical work is required.

SUMMARY

Brief treatment of unfertilized starfish eggs with hypertonic sea water increases the rate of activation by subsequent exposure to butyric acid. This effect may be described as a sensitization to the activating influence of the acid. The curve representing the relation between the duration of the hypertonic treatment and the rate of acid-activation resembles the bimolecular reaction isotherm.

In general the experimental facts of acid-activation (and of heat-activation) are consistent with the following simplified schema. Activation is the physiological result of the accumulation of a reaction product, called (provisionally) "the activating substance" (substance *A*) which is formed by the chemical union of two chief compounds. One

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[9] Sodium fluoride also, in experiments with .002, .004, .008 and .01 per cent solutions in sea water, was found not to interfere with heat-activation. In one experiment 90 per cent of eggs formed blastulae after 6 minutes in sea water at 32° containing .01 per cent NaF.

Dr. E. S. Guzman Barron informs me that he has found iodoacetate to have no depressant action but, if anything, a slight promoting action upon oxygen-consumption in *Arbacia* eggs.

of these, called (after the analogy with light-sensitization) the "sensitizing substance" (substance S), is already present in the egg; the other, the "acid-product" (substance B), is set free (*e.g.*, by hydrolysis) under the influence of the activating acid. The equation $S + B = A$ symbolizes this hypothetical activation-reaction.

Treatment with hypertonic sea water increases the concentration of S at the site of the activation-reaction, through the abstraction of water and promotion of dehydrolytic synthesis. The resulting increase in the rate of acid-activation is thus explained. Treatment with dilute sea water should theoretically have the reverse effect, but in this case the experimental evidence is conflicting.

The acid-product B appears to be formed at a rate proportional (through a certain range) to the concentration of acid; it is regarded as being set free by the hydrolysis of some normal egg-component at a rate controlled by the cH. The special hypothesis that the hydrolyzed compound is a phosphagen ester has, however, received no support from test experiments.

The hypothesis that heat-activation is an effect of the formation of lactic acid by glycolysis is also not supported by experiments with iodoacetate and fluoride.

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INDEX

ACTIVATION, artificial, of starfish eggs, as influenced by hypertonic and hypotonic sea water, 361.
Air turbine, for high speed centrifuging of biological material, 48.
Amines, toxicity for Amoeba proteus, effect of H-ion concentration, 276.
Amylase, concentrations in marine fishes, 133.
Amylases, as influenced by temperature, in cold- and warm-blooded animals, 330.
Anesthetics, effect on surface precipitation reaction, 264.
Anoplodactylus lentus Wilson, colored corpuscles of blood, 62.
Anuran tympanic membrane, stability and growth following larval involution, 38.
Asterias forbesii, electrokinetic studies, 247.

BARNES, T. Cunliffe. Further observations on the salt requirements of Ligia in Bermuda, 124.
Bases, excess, in sea water, as affected by land drainage, 97.
Bats, embryo, nema parasites in, 357.
Bernstein, Alan. *See* Howland and Bernstein, 276.
Binary fission, nuclei of various ciliates from fresh-water mussels during, 286.
Biparental males and biparental ratios, in Habrobracon, 166.
Birnie, James H. Regeneration of the tail-fins of Fundulus embryos, 316.
Blood, colored corpuscles of, in purple sea spider, 62.
Bostian, C. H. Biparental males and biparental ratios in Habrobracon, 166.

CALCIUM, mobilization of, during development, 351.
Cardiac ganglion of Limulus polyphemus, paradox phenomena in, 102.
Cellulose-digesting flagellate, cultivation of, 182.
Centrifugal force, effects on ectoplasmic layer and nuclei of fertilized sea urchin eggs, 228.
Centrifuged egg of Cumingia, hyaline zone, 257.
Centrifuging biological material at high speeds, with air turbine, 48.
Cerebratulus lacteus, electrokinetic studies, 247.
Chamberlain, Thomas K. The glochidial conglutinates of the Arkansas fanshell Cyprogenia aberti (Conrad), 55.
Chao, Iping. Paradox phenomena in the cardiac ganglion of Limulus polyphemus, 102.
Chesley, Leon C. The concentrations of proteases, amylase, and lipase in certain marine fishes, 133.
——, — —. The influence of temperature upon the amylases of cold- and warm-blooded animals, 330.
Chlorogonium and Chilomonas, trophic nature of, 1.
Ciliates from fresh-water mussels, structure and neuromotor system, 69.
—— — —— ——, nuclei during binary fission, 286.
Coe, Wesley R. Analysis of the regenerative processes in nemerteans, 304.
Coonfield, B. R. Coördination and movement of the swimming plates of Mnemiopsis leidyi, Agassiz, 10.
Costello, Donald P. The hyaline zone of the centrifuged egg of Cumingia, 257.
Cultivation of cellulose-digesting flagellate and other termite protozoa, 182.
Cumingia tellinoides, electrokinetic studies, 247.
Cyprogenia aberti (Conrad), glochidial conglutinates, 55.

DAN, KATSUMA. Electrokinetic studies of marine ova. II. Cumingia tellinoides, Asterias forbesii, Echinarachnius parma, Nereis limbata, and Cerebratulus lacteus, 247.

DAWSON, ALDEN B. The colored corpuscles of the blood of the purple sea spider, Anoplodactylus lentus Wilson, 62.

Division, longitudinal, in Hydra, 326.

Dogfish, hematological studies in, 115.

Drainage, from land, effects on excess bases of sea water, 97.

ECHINARACHNIUS parma, electrokinetic studies, 247.

Ectoplasmic layer, effects of centrifugal force on, in fertilized sea urchin eggs, 228.

Egg-trinuclearity, in Habrobracon, 145.

Elasmobranch fishes, control of dermal melanophores, 30.

Electrokinetic studies, marine ova, 247.

Eosin, concentration of, and photodynamic effect on tentacles of terebellid worm, 346.

FERTILIZATION, artificial, and induced ovulation in the frog, 22.

Fundulus, regeneration of tail-fins in embryos, 316.

GLAZER, OTTO, and EDMUND PIEHLER. The mobilization of calcium during development, 351.

Glochidial conglutinates of Cyprogenia aberti (Conrad), 55.

GREB, RAYMOND J. *See* Whiting, Greb, and Speicher, 152.

HABROBRACON, biparental males and biparental ratios, 166.

——, egg-trinuclearity, 145.

HAMLETT, G. W. D. Nema parasites in embryo bats, 357.

HARVEY, ETHEL BROWNE. Effects of centrifugal force on the ectoplasmic layer and nuclei of fertilized sea urchin eggs, 228.

HARVEY, E. NEWTON. Biological effects of heavy water, 91.

—— — ——. The air turbine for high speed centrifuging of biological material, together with some observations on centrifuged eggs, 48.

HEDENIUS, ASTRID. *See* Svedberg and Hedenius, 191.

HELFF, O. M. Studies on amphibian metamorphosis. XIII. Stability and growth of anuran tympanic membrane following larval involution, 38.

Hematological studies in dogfish, 115.

H-ion concentration as factor in toxicity of amines for Amoeba proteus, 276.

HEILBRUNN, L. V. The effect of anesthetics on the surface precipitation reaction, 264.

HOWLAND, RUTH B., and ALAN BERNSTEIN. H-ion concentration as a factor in the toxicity of amines for Amoeba proteus, 276.

Hyaline zone of centrifuged egg of Cumingia, 257.

Hydra, longitudinal division in, 326.

INVOLUTION, larval, followed by stability and growth of anuran tympanic membrane, 38.

KIDDER, GEORGE W. Studies on the ciliates from fresh-water mussels. I. The structure and neuromotor system of Conchophthirius anodontae Stein, C. curtus Engl., and C. magna sp. nov., 69.

——, —— —. Studies on the ciliates from fresh-water mussels. II. The nuclei of Conchophthirius anodontae Stein, C. curtus Engl., and C. magna Kidder, during binary fission, 286.

LIGIA, salt requirements of, in Bermuda, 124.

LILLIE, RALPH S. The influence of hypertonic and hypotonic sea water on the artificial activation of starfish eggs, 361.

Limulus polyphemus, paradox phenomena in the cardiac ganglion, 102.

Lipase, concentration of, in marine fishes, 133.

LOEFER, JOHN B. The trophic nature of Chlorogonium and Chilomonas, 1.

MELANOPHORES, dermal, control of, in elasmobranch fishes, 30.
Metamorphosis, and growth of anuran tympanic membrane, 38.
MITCHELL, PHILIP H., and JULIAN P. SOLINGER. The effects of land drainage upon the excess bases of sea water, 97.
Mnemiopsis leidyi, Agassiz, coördination and movement of swimming plates, 10.

NEMA parasites, in embryo bats, 357.
Nemerteans, regenerative processes in, 304.
Nereis limbata, electrokinetic studies, 247.
Nuclei, effects of centrifugal force on, in fertilized sea urchin eggs, 228.
—— of ciliates during binary fission, 286.

OVA, marine, electrokinetic studies of, 247.
Ovulation, induced, and artificial fertilization in the frog, 22.

PARASITES, nema, in embryo bats, 357.
PARKER, G. H., and HELEN PORTER. The control of the dermal melanophores in elasmobranch fishes, 30.
PIEHLER, EDMUND. *See* Glazer and Piehler, 351.
PORTER, HELEN. *See* Parker and Porter, 30.
Proteases, concentrations, in marine fishes, 133.

REGENERATION, of tail, as affected by Ringer's solution, in tadpoles of Rana palustris, 7.
——, of tail-fins of Fundulus embryos, 316.
——, processes of, in nemerteans, 304.
Respiratory proteins, sedimentation constants, 191.
REZNIKOFF, PAUL, and DOROTHY GROUT REZNIKOFF. Hematological studies in dogfish (Mustelus canis), 115.
Ringer's solution, effect on tail-regeneration, in Rana palustris, 7.
ROUDABUSH, ROBERT L. Present status of longitudinal division in Hydra, 326.
RUGH, ROBERTS. Induced ovulation and artificial fertilization in the frog, 22.

SALT requirements of Ligia in Bermuda, 124.
SAYLES, LEONARD P. Effect of Ringer's solution on tail-regeneration in the tadpole of Rana palustris, 7.
SCHMUCK, M. LOUISE. The male somatic chromosome group in Sciara pauciseta, 224.
Sea urchin eggs, fertilized, effects of centrifugal force on ectoplasmic layer and nuclei, 228.
Sea water, effect of land drainage on excess bases, 97.
— ——, hypertonic and hypotonic, influence on artificial activation of starfish eggs, 361.
Sedimentation constants of respiratory proteins, 191.
Sex-intergrade, new type of, 152.
SOLINGER, JULIAN P. *See* Mitchell and Solinger, 97.
Somatic chromosome group, male, in Sciara pauciseta, 224.
SPEICHER, B. R. *See* Whiting, Greb, and Speicher, 152.
Starfish eggs, influence on artificial activation of hypertonic and hypotonic sea water, 361.
Surface precipitation reaction, effect of anesthetics on, 264.
SVEDBERG, THE, and ASTRID HEDENIUS. The sedimentation constants of the respiratory proteins, 191.
Swimming plates, coördination and movement of, in Mnemiopsis leidyi, Agassiz, 10.

TAIL-fins, regeneration of, in Fundulus embryos, 316.
Temperature, influence on amylases of cold- and warm-blooded animals, 330.
Tentacles, of Terebella magnifica W., structure and reactions of, 339.
——, photodynamic effect of eosin on, of terebellid worm, 346.
Terebella magnifica W., structure and reactions of tentacles, 339.

TRAGER, WILLIAM. The cultivation of a cellulose-digesting flagellate, Trichomonas termopsidis, and of certain other termite protozoa, 182.
Trophic nature of Chlorogonium and Chilomonas, 1.

WATER, heavy, biological effects, 91.
WELSH, JOHN H. The concentration of eosin and the photodynamic effect on tentacles of a terebellid worm, 346.
——, —— —. The structure and reactions of the tentacles of Terebella magnifica W., 339.
WHITING, P. W. Egg-trinuclearity in Habrobracon, 145.
——, —., RAYMOND J. GREB, and B. R. SPEICHER. A new type of sex-intergrade, 152.

1934
43249

Volume LXVI Number 1

THE
BIOLOGICAL BULLETIN

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FEBRUARY, 1934

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year. Single numbers, $1.75. Subscription per volume (3 numbers), $4.50.

Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa. Agent for Great Britain: Wheldon & Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W.C. 2.

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Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

CONTENTS

Page

LOEFER, JOHN B.

The Trophic Nature of Chlorogonium and Chilomonas...... 1

SAYLES, LEONARD P.

Effect of Ringer's Solution on Tail-Regeneration in the Tadpole of Rana palustris.............................. 7

COONFIELD, B. R.

Coördination and Movement of the Swimming Plates of Mnemiopsis leidyi, Agassiz 10

RUGH, ROBERTS

Induced Ovulation and Artificial Fertilization in the Frog ... 22

PARKER, G. H., AND HELEN PORTER

The Control of the Dermal Melanophores in Elasmobranch Fishes 30

HELFF, O. M.

Studies on Amphibian Metamorphosis. XIII. Stability and growth of anuran tympanic membrane following larval involution... 38

HARVEY, E. NEWTON

The Air Turbine for High Speed Centrifuging of Biological Material, together with some Observations on Centrifuged Eggs.. 48

CHAMBERLAIN, THOMAS K.

The Glochidial Conglutinates of the Arkansas Fanshell Cyprogenia aberti (Conrad)............................. 55

DAWSON, ALDEN B.

The Colored Corpuscles of the Blood of the Purple Sea Spider, Anoplodactylus lentus Wilson....................... 62

KIDDER, GEORGE W.

Studies on the Ciliates from Fresh-water Mussels, I....... 69

Volume LXVI Number 2

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APRIL, 1934

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year. Single numbers, $1.75. Subscription per volume (3 numbers), $4.50.

Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa. Agent for Great Britain: Wheldon & Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W.C. 2.

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CONTENTS

Page

HARVEY, E. NEWTON
Biological Effects of Heavy Water 91

MITCHELL, PHILIP H., AND JULIAN P. SOLINGER
The Effects of Land Drainage upon the Excess Bases of Sea Water 97

CHAO, IPING
Paradox Phenomena in the Cardiac Ganglion of Limulus polyphemus 102

REZNIKOFF, PAUL, AND DOROTHY GROUT REZNIKOFF
Hematological Studies in Dogfish (Mustelus canis) 115

BARNES, T. CUNLIFFE
Further Observations on the Salt Requirements of Ligia in Bermuda 124

CHESLEY, LEON C.
The Concentrations of Proteases, Amylase, and Lipase in Certain Marine Fishes 133

WHITING, P. W.
Egg-Trinuclearity in Habrobracon 145

WHITING, P. W., RAYMOND J. GREB, AND B. R. SPEICHER
A New Type of Sex-Intergrade 152

BOSTIAN, C. H.
Biparental Males and Biparental Ratios in Habrobracon 166

TRAGER, WILLIAM
The Cultivation of a Cellulose-Digesting Flagellate, Trichomonas termopsidis, and of certain other Termite Protozoa 182

SVEDBERG, THE, AND ASTRID HEDENIUS
The Sedimentation Constants of the Respiratory Proteins . . . 191

SCHMUCK, M. LOUISE
The Male Somatic Chromosome Group in Sciara pauciseta 224

HARVEY, ETHEL BROWNE
Effects of Centrifugal Force on the Ectoplasmic Layer and Nuclei of Fertilized Sea Urchin Eggs 228

Volume LXVI Number 3

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CONTENTS

Page

DAN, KATSUMA
Electrokinetic Studies of Marine Ova. II. Cumingia tellinoides, Asterias forbesii, Echinarachnius parma, Nereis limbata, and Cerebratulus lacteus 247

COSTELLO, DONALD P.
The Hyaline Zone of the Centrifuged Egg of Cumingia 257

HEILBRUNN, L. V.
The Effect of Anesthetics on the Surface Precipitation Reaction.. 264

HOWLAND, RUTH B., AND ALAN BERNSTEIN
H-Ion Concentration as a Factor in the Toxicity of Amines for Amoeba proteus 276

KIDDER, GEORGE W.
Studies on the Ciliates from Fresh Water Mussels. II. The nuclei of Conchophthirius anodontae Stein, C. curtus Engl., and C. magna Kidder, during binary fission 286

COE, WESLEY R.
Analysis of the Regenerative Processes in Nemerteans 304

BIRNIE, JAMES H.
Regeneration of the Tail-Fins of Fundulus Embryos 316

ROUDABUSH, ROBERT L.
Present Status of Longitudinal Division in Hydra 326

CHESLEY, LEON C.
The Influence of Temperature upon the Amylases of Cold- and Warm-blooded Animals 330

WELSH, JOHN H.
The Structure and Reactions of the Tentacles of Terebella magnifica W. 339

WELSH, JOHN H.
The Concentration of Eosin and the Photodynamic Effect on Tentacles of a Terebellid Worm 346

GLASER, OTTO, AND EDMUND PIEHLER
The Mobilization of Calcium during Development 351

HAMLETT, G. W. D.
Nema Parasites in Embryo Bats 357

LILLIE, RALPH S.
The Influence of Hypertonic and Hypotonic Sea Water on the Artificial Activation of Starfish Eggs 361

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